

**TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)**

10/049316
7529/1F590-US1

INTERNATIONAL APPLICATION NO.

INTERNATIONAL FILING DATE

PRIORITY DATE CLAIMED

PCT/US00/22106

10
10 August 2000

13 August 1999

TITLE OF INVENTION

PROTECTIVE ANTIGEN OF EPSTEIN BARR VIRUS

APPLICANT(S) FOR DO/EO/US

Ralph M. STEINMAN and Christian MUENZ

Applicant herewith submits to the United States Designated/Elected office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371 (f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371 (b) and PCT Articles 22 and 39 (1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau
 - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371 (c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c) (3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98 (with 11 references).
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney an/or address letter.
16. ☐ Other items or information:

EXPRESS MAIL CERTIFICATE

Date **2/8/02** Label No. **42167723530US**

I hereby certify that, on the date indicated above, this paper or fee was deposited with the U.S. Postal Service & that it was addressed for delivery to the Assistant Commissioner for Patents, Washington, DC 20231 by "Express Mail" service to Addressee's service.

O Biek
Name (Print)

[Signature]
Signature

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.50)

INTERNATIONAL APPLICATION NO.:

Attorney's Docket Number

10/049316

PCT/US00/22106

7529/22106 / F590usj

17. [x] The following fees are submitted:

Basic National Fee (37 CFR 1.492 (a)(1)-(5)): Search Report has been prepared by the EPO <input type="checkbox"/> or JPO <input type="checkbox"/>	\$890.00
International preliminary examination fee paid to USPTO (37 CFR 1.482)XXX.....	\$710.00
No international preliminary examination fee paid to USPTO (37 CFR 4.482) but international search fee paid to USPTO (37 CFR 1.445 (a) (2)...	\$740.00
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....	\$1,040.00
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)....	\$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =

\$710.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(e)).

\$

Claims	Number Filed	Number Extra	Rate		
Total Claims	19-20	0	0	X \$18.00	\$00
Independent Claims	3 -3	0	0	X \$84.00	\$00
Multiple dependent claim(s) (if applicable)		+280			\$
TOTAL OF ABOVE CALCULATIONS =				\$710.00	
Reduction by 1/2 for filing by small entity, if applicable.				\$355.00	
SUBTOTAL =				\$355.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				+	\$
TOTAL NATIONAL FEE =				\$00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)), the assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property				+	\$40.00
TOTAL FEES ENCLOSED =				\$395.00	
				Amount to be refunded	\$
				charged.	\$

a. [X] A check in the amount of \$395.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No.04-0100 in the amount of \$ to cover the above fees.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 04-0100. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Paul F. Fehlner, Ph.D.
Darby & Darby P.C.
805 Third Avenue
New York, New York 10022-7513

SIGNATURE

Paul F. Fehlner

NAME Paul F. Fehlner, Ph.D.

REGISTRATION NO. 35,135

10/049316

JC13 Rec'd PCT/PTO 08 FEB 2002

EXPRESS MAIL CERTIFICATE

Date 2/8/02 Label No. 6276772353015

I hereby certify that, on the date indicated above, this paper or fee was deposited with the U.S. Postal Service & that it was addressed for delivery to the Assistant Commissioner for Patents, Washington, DC 20231 by "Express Mail" and to Addressee's name.

PLEASE CHARGE ANY DEFICIENCY UP TO \$300.00 OR CREDIT ANY EXCESS IN THE FEES DUE WITH THIS DOCUMENT TO OUR DEPOSIT ACCOUNT NO. 04-0100

Name (Print)

Signature

Customer No.:



07278

PATENT TRADEMARK OFFICE

Docket No.: 7529/1F590-US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Ralph M. STEINMAN and Christian MUENZ

Serial No.: t/b/a (U.S. National Phase of International Application

No. PCT/US00/22106 filed August 10, 2000)

Filed: Concurrently Herewith

For: PROTECTIVE ANTIGEN OF EPSTEIN BARR VIRUS

PRELIMINARY AMENDMENT

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Box PCT
Attn.: DO/EO/US

Sir:

Prior to examination, please amend the above-identified application as follows:

In the Specification:

Page 1, above line 8, insert the following paragraph:

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a U.S. national phase application under 35 U.S.C. §371 based upon co-pending International Application NO. PCT/US00/22106 filed August 10, 2000, which claims the benefit of priority of U.S. Provisional Application Serial NO. 60/148,971 filed August 13, 1999. The entire disclosures of the prior applications are incorporated herein by reference. The international application was published in the English language on February 22, 2001 under Publication No. W001/12215.


REMARKS

The specification has been amended to provide proper reference to the prior related applications.

Entry of this Amendment is respectfully requested.

Respectfully submitted,

Dated: February 8, 2002


Paul F. Fehlner, Ph.D.
Registration No. 35,135
Attorney for Applicants

DARBY & DARBY
805 Third Avenue
New York, NY 10022
(212) 527-7700

10/049316

PROTECTIVE ANTIGEN OF EPSTEIN BARR VIRUS

This work was supported in part by National Institutes of Health Grant
5 No. K12-HD00850 and NIAID grants AI40045 and AI40874. Accordingly, the
United States government may have certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to the identification of a vaccine to
10 prevent or treat Epstein Barr Virus infection. In particular, the EBNA-1 antigen was
identified as a vaccine antigen. Exposure to EBNA-1 can be exploited for its
immunoprotective effect in humans and in animals.

BACKGROUND OF THE INVENTION

15 EBV is a human gamma herpesvirus with a tropism for B lymphocytes
(Kieff and Liebowitz, in Virology, eds. Fields, B.N., Knipe, D.M. *et al.*, p. 1889-1919,
Raven Press, Ltd.: New York, 1990). Greater than 95% of the adult population carry
EBV as a lifelong asymptomatic infection. However EBV has strong growth
transforming capacities (Klein, Cell, 77:791-3, 1994), transforming B cells and
20 presumably other cell types in a spectrum of EBV-associated malignancies including
Hodgkin's lymphoma, nasopharyngeal carcinoma, T cell lymphoma, gastric
carcinoma, and uterine leiomyosarcoma.

Three specific EBV genes are critical for tumorigenesis and induce cell
proliferation as well as resistance to apoptosis (Gregory, *et al.*, Nature, 349:612-4,
25 1991). EBNA-1 links as a dimer the viral origin of replication and the host cell DNA
and ensures episomal replication during B cell growth (Bochkarev, *et al.*, Cell,
84:791-800; Shah, *et al.*, J. Virol., 66:3355-62, 1992). The two latent membrane
proteins (LMP) have different roles. The C-terminal part of LMP1 can act as a direct

-2-

oncogene (Wang, *et al.*, Cell, 43:831-40, 1985) by mimicking CD40-mediated B cell activation (Busch and Bishop, J. Immunol., 162:2555-2561, 1999). Thus, LMP1 engages signaling proteins for the tumor necrosis factor receptor family (Mosialos, *et al.*, Cell, 80:389-99, 1995) and protects against apoptosis by induction of bcl-2 (Henderson, *et al.*, Cell, 65:1107-15, 1991). LMP2 mimics B cell receptor signaling by constitutively engaging syk and lyn, protein tyrosine kinases (Caldwell, *et al.*, Immunity, 9:405-11, 1998). These three proteins appear to be the exclusive EBV genes that are expressed in most EBV-induced tumors (Miller, *et al.*, in Virology, eds. Fields, B.N., Knipe, D.M. *et al.*, p. 1921-1958, Raven Press, Ltd.: New York, 1990).

10 In Burkitt's lymphoma, only EBNA-1 is required for EBV persistence, since transformation is achieved by an additional mechanism involving c-myc uncoupling through chromosomal translocation (Klein, *supra*).

The reason why most carriers of EBV avoid transformation remains to be elucidated. Immunity to EBNA-1 *a priori* could provide resistance to transformed cells, but it has proven difficult to detect specific T cell responses to this essential protein for EBV persistence. In fact, EBNA-1 blocks its own processing for MHC class I presentation (Blake, *et al.*, Immunity, 7:791-802, 1997). This has been attributed to a deficit in proteasomal processing, caused by the N-terminal GA repeat domain (Levitskaya, *et al.*, Nature, 375:68508, 1995). A similar GA stretch prevents

20 I κ B α degradation by the proteasome (Sharipo, *et al.*, Nat Med., 4:939-44, 1998). Other EBV latency gene products are the focus of a strong MHC class I restricted CTL response, especially EBNA3A, 3B, and 3C (Steven, *et al.*, J. Exp. Med., 184:1801-13, 1996). However, the EBNA3 proteins are not expressed in most of the EBV-associated tumors mentioned above, and instead are expressed in cultured

25 transformed lines (B-LCL) and lymphoproliferative syndromes in immunosuppressed patients. CD8⁺ CTL responses to tumor-associated LMP1 (Khanna, *et al.*, Eur. J. Immunol., 28:451-8, 1998) and LMP2 (Lee, *et al.*, Eur. J. Immunol., 26:1875-83, 1996) proteins have been detected, but only occasionally.

It is becoming apparent that the development and persistence of effective CD8⁺ CTLs are dependent on CD4⁺ T cell help (Kalams and Walker, J. Exp.

30

-3-

Med., 188:2199-204, 1998). Recognition of EBV products by CD4⁺ T cells has not been investigated in the same detail as the CD8⁺ response (Rickinson and Moss, Ann. Rev. Immunol., 15:405-31, 1997). Dendritic cells (DCs) are potent antigen presenting cells for CD4⁺ and CD8⁺ T cell immunity (Banchereau and Steinman, Nature, 392:245-52, 1998).

Thus, the efforts to identify a protective antigen in Epstein Barr Virus have been inconclusive, and it is unknown whether a single or multiple antigens are necessary to provide complete protection from infection. Furthermore, EBNA-1 is not believed to elicit protective immunity to the virus.

SUMMARY OF THE INVENTION

The present invention advantageously identifies an immuno-protective antigen from EBV, which can be used in vaccine and immunotherapy approaches to preventing or treating EBV infection in humans.

In one embodiment, the invention provides a vaccine comprising an immunogenic EBNA-1 polypeptide and an adjuvant acceptable for use in a human. The immunogenic EBNA-1 polypeptide can be a fusion protein of EBNA-1 and a heterologous amino acid sequence.

In another embodiment, the invention provides an expression vector for expression in humans comprising a sequence encoding an immunogenic EBNA-1 polypeptide, operably associated with an expression control sequence. A preferred vector of the invention preferentially targets dendritic cells. The invention specifically contemplates viral vectors, such as a vaccinia virus vector, Fowl pox, AV-pox, and modified vaccinia Ankara (MVA) virus.

Also provided are methods for protecting a subject from infection by Epstein Barr Virus. One such method comprises delivering an immunologically effective amount of an immunogenic EBNA-1 polypeptide to the subject. Another such method comprises delivering an immuno-protective amount of the expression vector of the invention to the subject. Preferably such an expression vector targets dendritic cells *in vivo*. These methods are useful to prevent or treat an EBV-

-4-

associated neoplasm, such as (but not limited to) nasopharyngeal carcinoma.

The present invention is further explained and exemplified in the following Detailed Description and Example.

5

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1A, 1B, 1C, 1D, 1E, and 1F. EBNA-1 is preferentially recognized by CD4⁺ T cells. Blast formation by CD4⁺ T cells (CD4-FITC) was monitored by flow cytometry. The forward scatter indicates the size of the cells. Cultures of CD2⁺CD8⁺ T cells, stimulated with autologous DC infected with vaccinia virus constructs, were analyzed. **A.** Culture stimulated with vvEBNA-1ΔGA infected DCs and restimulated with vvTK⁻ infected DCs. **B.** Blasting of a culture stimulated with vvEBNA-1ΔGA infected DCs and restimulated with vvEBNA-1ΔGA infected DCs. **C.** Culture stimulated with vvEBNA3A infected DCs and restimulated with vvTK⁻ infected DCs. **D.** T cells were stimulated and restimulated with vvEBNA3A infected DCs. **E.** Culture stimulated and restimulated with vvTK⁻ infected DCs to evaluate the background of vaccinia stimulation. **F.** CD2⁺CD8⁺ T cells responding to influenza virus infected DCs as positive control. All cultures were prepared from the same donor (No. 5 in Table 1). Percent of blasted subpopulations (arrows) are indicated.

20

FIGURE 2A, 2B, 2C, and 2D. Recognition of EBNA-1 provided to DCs by endogenous and exogenous pathways. **A.** CD4⁺ (CD4-FITC) versus CD8⁺ (CD8-PE) content. **B.** CD56⁺ (CD56-PE) content. **C.** IFNγ spot forming cells / 10⁵ cells stimulated with recombinant baculovirus expressed EBNA-1 protein loaded DCs (DC + bEBNA-1) and without loading (DC). **D.** Spot formation of the line upon incubation with vvTK⁻ infected DCs (DC + vvTK⁻), vvEBNA-1ΔGA infected DCs (DC + vvEBNA-1ΔGA), vvT7 infected DCs (DC + vvT7), vvEBNA-1 infected DCs (DC + vvEBNA-1) and vvEBNA-1/vvT7 double infected DCs (DC + vvEBNA-1 + vvT7). In the same figure the MHC restriction is analyzed using the antibodies L243, αHLA-DR, (+L243) and B-H9, αHLA class I, (+B-H9) for blocking. In addition, spot

25

-5-

formation upon stimulation with the HLA-DR4⁺ B-LCL, LRM (LRM), vs. the HLA-DR4⁺ B-LCL, LG2 (LG2), is shown.

FIGURE 3A and 3B. Proliferative responses of the CM171198 cell line to various EBNA-1 expressing targets. A: DCs (DC only) and T cells (T cells only) show low background proliferation. When mixed (0) there is some proliferation that can be increased upon infection with a recombinant vaccinia virus construct expressing EBNA-1 (vvEBNA-1ΔGA) but not vector alone (vvTK⁻). External loading of DCs with recombinant EBNA-1 proteins from *E. coli* (eEBNA-1) and baculovirus/insect cell (bEBNA-1) expression systems also augments the proliferation, while there is no reactivity against an *E. coli* derived control protein (eControl). B: B-LCL sharing the HLA-DR4 allele with the CM171198 cell line induce proliferation (LRM and LCL-BM) while HLA-DR4 mismatched B-LCL do not (LG2 and LCL-DC).

FIGURE 4A, 4B, 4C, 4D, 4E, 4F, 4G, and 4I. Cytotoxic activity and EBV latent antigen specificity of T cell subsets. The left column (A-C) shows the CD8 vs. CD4 FACS stainings of three PBMC responder populations: (A) CD2⁺PBMC, (B) CD8⁺CD2⁺PBMC and (C) CD4⁺CD2⁺PBMC. The middle column (D-F) displays the observed lysis of autologous LCL (LCL-JT; solid circles) and T2 cells (T2; open circles) by these responders. In the right column (G-I) the EBV latent antigen specificity of the three responder populations was investigated in an ELISPOT assay (E1: vvEBNA-1ΔGA, E2: vvEBNA2, E3B: vvEBNA3B, E3C: vvEBNA3C, L1: vvLMP1, L2a: vvLMP2A, B1: vvBMLF1, LCL: LCL-JT).

FIGURE 5A, 5B, 5C, 5D, 5E, and 5F. HLA-DR restriction and EBNA-1 recognition by CTL subsets. The effectors, as shown on the top, were either CD2⁺PBMC (A, D), CD8⁺CD2⁺PBMC (B, E) or CD4⁺CD2⁺PBMC (C, F). The top row (A-C) shows cytolysis of autologous B-LCL (LCL-JT) in the presence (open circles, dotted line) or absence (solid circles) of 5μg/ml L243, αHLA-DR antibody (LCL-JT + L243). T2 cells (solid triangles) were used as a control. The bottom row (D-F) shows lytic activity against autologous B-LCL (LCL-JT; open triangles) in comparison to autologous DCs pulsed with *E. coli* derived control protein (eControl;

-6-

solid circles), *E. coli* derived EBNA-1 protein (eEBNA-1; open circles) or baculovirus/insect cell derived EBNA-1 protein (bEBNA-1; solid triangles).

FIGURE 6A and 6B. Specificity of CD4⁺ CTL clones. B-LCL were generated from samples of 2 leukocyte concentrates. Cryopreserved CD8⁺ CD2⁺PBMC were then stimulated for 2 weeks with these autologous B-LCL under limiting dilution. Afterwards the wells were split and tested in ⁵¹Cr release assays against vvEBNA-1ΔGA (open bar) or vvTK⁻ (dark solid bar) infected DCs as well as autologous B-LCL (gray solid bar) and LCL721.221 (second open bar), a HLA class I NK target. 11 clones recognizing EBNA-1 epitopes on EBV transformed B cells are shown. They were derived from 2 leukocyte concentrates in 2 independent experiments (A,B).

FIGURE 7A and 7B. EBNA-1-specific T cell responses are dominated by T_H1 cytokine secretion. CD4⁺ T cells were stimulated with dendritic cells infected with vvEBNA-1ΔGA or vvTK⁻ (negative control) and tested for their secretion of IFN-γ (A) or IL-4 (B) on the day of T cell isolation (day 1) and after one week expansion (day 7). Values shown are the mean of triplicates and were derived by the subtraction of the negative control from the number of EBNA-1-specific spots. The values of the vvTK⁻ control ranged from 0 to 105. A vvEBNA-1ΔGA response was considered significant if it were at least ten spots above vvTK⁻ negative control and at least twice that of the negative control.

FIGURE 8A and 8B. EBNA-1-specific responses can be detected at very low doses of antigen. A recombinant EBNA-1 protein or control proliferating cell nuclear antigen (PCNA) protein was eluted from *E. coli* expressing vectors. A. Proteins were dialyzed overnight and tested for purity with SDS PAGE. The recovered rEBNA-1 protein was tested for specificity by Western blot using an anti-EBNA-1 antibody MAB8173. The antibody AD1.1.10 recognizes a histidine tag which is contained in the rEBNA-1 protein. B. vvEBNA-1ΔGA-infected DCs were used to expand CD4⁺ T cells in a one week culture. The expanded T cells were restimulated using DCs pulsed with the indicated concentration of rEBNA-1 protein or rPCNA control protein and read-out with ELISPOT. The rEBNA-1 protein was

-7-

added to the DCs during the maturation phase (day 6-8) of the DC culture. The inset graph shows the ELISPOT results of CD4⁺ T cells expanded with vvEBNA-1ΔGA-infected DCs for one week and restimulated with either vvEBNA-1ΔGA-infected DCs or vvTK⁻ control.

5 **FIGURE 9A, 9B, 9C, 9D, 9E, and 9F.** EBNA-1-specific cells from fresh blood are CD4⁺ T cells. Freshly isolated PBMCs were stimulated with vvEBNA-1ΔGA-infected dendritic cells for either 7 hours for maximal production of IFN γ , or 18 hours for IL-4. They were then positively selected based on the secretion of either IFN γ or IL-4. IFN γ cell lines were established in 6/6 donors and IL-4 cell
10 lines in 3/6 donors. Shown are control stains (A,B,C) CD4 and CD8 stains (D, E, F) of three IFN γ lines analyzed by FACS. The gates were set on the lymphocyte population and on living cells as determined by propidium iodide staining.

FIGURE 10A, 10B, 10C, 10D, and 10E. EBNA-1-specific T_H1 cells and not T_H2 cells lyse EBNA-1-expressing DCs. Cell lines were isolated from
15 PBMCs stimulated with vvEBNA-1ΔGA-infected DCs for either 7 hours (IFN γ) or 18 hours (IL-4). The cells were then positively selected based on the secretion of IFN γ or IL-4. EBNA-1-specific IFN γ and IL-4 producing cells were isolated from 6/6 donors and 3/6 donors, respectively. These cells were expanded with weekly restimulations of irradiated vvEBNA-1ΔGA-infected DCs alternating with DCs pulsed with a
20 rEBNA-1 protein. **A, B.** Cells were restimulated with vvEBNA-1ΔGA-infected (open bar) or vvTK⁻ infected (solid bar) DCs and tested for either IFN γ or IL-4 secretion after three weeks of expansion. The IFN γ -secreting cell line isolated from LD03 which is representative of all T_H1 cell lines is shown in panel A, and a representative EBNA-1-specific IL-4 secreting cell line from LD01 is illustrated in panel B. **C, D.**
25 Cells were then tested for their ability to lyse vvEBNA-1ΔGA-infected DCs by ⁵¹Cr release assay. **C.** The results of the ⁵¹Cr release from LD03 cell line are shown with graded effector to target ratios and are representative of all six established IFN γ cell lines. **D.** Results shown are from a cell line established from LD01 and are representative of all IL-4-secreting cell lines isolated. **E.** CTL assay results at an
30 effector to target ratio of 10:1 are shown for 5 IFN γ cell lines and 3 IL-4 lines for

-8-

three sources of EBNA-1 antigen (vvEBNA-1ΔGA, rEBNA-1 or the physiologically expressed protein in BLCL) as compared to controls (vvTK, rPCNA control protein, T2 cells, respectively). Different symbols are representative of each cell line with T_H1 cell lines in white and T_H2 cell lines in black. T_H1 and T_H2 cell lines isolated from the same donor share symbol shapes.

FIGURE 11A, 11B, 11C, 11D, and 11E. EBNA-1-specific antibodies are predominantly IgG1. Values are shown for each donor, with mean values indicated by black bars. The background levels in the absence of antigen (A) for each IgG subclass were subtracted from the results with the microbial proteins. IgG subclass distribution of antibodies specific to a rEBNA-1 protein are shown (B), as are the IgG subclass distribution to tetanus toxoid (C), candida (D) and mumps (E).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based, in part on unexpected discoveries concerning a protective antigen of Epstein Barr Virus (EBV). In most EBV seropositive adults, strong CD8⁺ cytotoxic T lymphocytes (CTL) responses have been demonstrated (Murray, *et al.*, J. Exp. Med., 1992, 176:157-168). However, these are preferentially directed toward the nuclear antigens, EBNA3A, 3B and 3C (Kieff, E., Epstein-Barr Virus and Its Replication. In Fields Virology. B.N. Fields, D.M. Knipe, and P.M. Howley, editors. 1996, Lippincott-Raven Publishers, Philadelphia. 2343-2396; Khanna, *et al.* J.Exp.Med., 1992, 176:169-176), which are not expressed in many EBV-associated malignancies. EBV-transformed cells exhibit one of three latency phenotypes distinguished from each other by the panel of expressed EBV antigens (Murray, *et al.*, 1992, *supra*). In latency I, *e.g.*, Burkitt's lymphoma, EBNA-1 alone is expressed. In latency II, exemplified by Hodgkin's lymphoma, LMP1 and LMP2 as well as EBNA-1 are expressed. Only in latency III immunoblastic lymphomas are the highly immunogenic EBNA3 genes expressed. Therefore, many EBV-associated malignancies do not seem to provide good targets for the human CD8⁺ T cell response to EBV latency gene products.

The evidence in the present invention suggests that, unexpectedly, the

-9-

Epstein-Barr virus (EBV) encoded nuclear antigen (EBNA-1) is an effective antigen for developing an EBV vaccine, particularly an anti-tumor vaccine. EBNA-1 immunity greatly reduces viral replication because this antigen is crucial for the persistence of the EBV episome in replicating EBV-transformed human B cells (Yates, *et al.*, Nature, 313:812-5, 1985). Therefore, all EBV-induced tumors express this foreign antigen. However, EBNA-1 protein is invisible to CD8⁺ cytotoxic T lymphocytes (CTLs). The gly-ala repeat domain prevents proteasome dependent processing and thus presentation on MHC class I (Levitskaya, *et al.*, *supra*). It has now been found that CD4⁺ T cells from most individuals do respond to EBNA-1. In fact, among EBV latent antigens that stimulate CD4⁺ cells, EBNA-1 is preferentially recognized. Recognition can occur via endogenous and exogenous processing of EBNA-1 onto MHC class II molecules of dendritic cells (DCs). The CD4⁺ response includes direct cytotoxicity of transformed B lymphocyte cell lines (B-LCL). Therefore, the immune system can recognize the EBNA-1 protein that is crucial for EBV persistence.

The type of CD4⁺ T cell also influences the response (reviewed in O'Garra, and Murphy, Curr. Opin. Immunol., 1994, 6:458-66. 20). T_H1 CD4⁺ cells secrete IFN γ and help in the development of cellular immunity, including the activation of macrophages. T_H2 CD4⁺ cells secrete IL-4 and IL-5, thereby stimulating eosinophils and antibody production. The expression of specific chemokine receptors on the T_H1 cells results in the migration of the T_H1 cells to the normal sites of inflammation. T_H2 cells migrate to sites more closely associated with allergic responses. CD4⁺ T cells also can kill targets (reviewed in Hahn, *et al.*, Immunol Rev., 1995, 146:57-79), primarily through Fas-FasL interactions. Cytotoxicity mainly has been found with T_H1 CD4⁺ cells (Erb, *et al.*, Cell Immunol., 1991, 135:232-44; Erb, *et al.*, J. Immunol., 1990, 144:790-795; Del Prete, *et al.*, J. Exp. Med., 1991, 174:809-13; Nishimura, *et al.*, J. Exp. Med., 1999, 190:617-628.), but a limited number of cytotoxic T_H2 CD4⁺ clones have been reported (Lancki, *et al.*, J. Immunol., 1991, 146:3242-9).

Evidence suggests that the EBNA-1-specific, CD4⁺ T cell response in

-10-

cells that are directly isolated from blood is predominantly a T_H1 response. Furthermore, the isotype of the EBNA-1 antibody response is skewed to the IgG1 subclass, reflecting T_H1 polarization *in vivo*. This result has important implications for a therapeutic vaccine for EBNA-1 based on the emerging evidence that T_H1 cells are important for resistance to viruses and tumors, and thus are key to long term immunity.

Immunotherapy of EBV with EBNA-1 provides significant advantages of safety and efficacy. Subunit vaccines ensure the greatest degree of safety because there is no opportunity for infection by the pathogen. This is always of some concern when immunizing with killed or attenuated virus. Furthermore, a single component minimizes adverse side effects, such as anaphylaxis or antigen cross reactivity, that may result from an unrelated antigen in a whole virus vaccine. Immunotherapy with dendritic cells charged with EBNA-1 has substantial therapeutic potential. Furthermore, because EBNA-1 is involved in EBV infection and tumorigenesis, it will elicit the most protective immune response to prevent or treat EBV infection and associated diseases or disorders. Another alternative vaccine approach is to define the DR-specific peptides in the EBNA-1 protein. Pulsing dendritic cells with these peptides, and then immunizing subjects with the pulsed dendritic cells would establish the protective effect of the vaccine strategy.

The term "vaccine" refers to a composition (protein or vector; the latter may also be loosely termed a "DNA vaccine", although RNA vectors can be used as well) that can be used to elicit protective immunity in a recipient. It should be noted that to be effective, a vaccine of the invention can elicit immunity in a portion of the population, as some individuals may fail to mount a robust or protective immune response, or, in some cases, any immune response. This inability may stem from the individual's genetic background or because of an immunodeficiency condition (either acquired or congenital) or immunosuppression (e.g., treatment with immunosuppressive drugs to prevent organ rejection or suppress an autoimmune condition). Efficacy can be established in animal models.

The term "immunotherapy" refers to a treatment regimen based on

-11-

activation of a pathogen-specific immune response. A vaccine can be one form of immunotherapy. Charging dendritic cells with EBNA-1 antigen, preferably with a stimulatory cytokine such as GM-CSF or Flt3 ligand *ex vivo* (followed by transplantation into the subject) or *in vivo* is also a form of immunotherapy.

5 The term "protect" is used herein to mean prevent or treat, or both, as appropriate, an EBV infection in a subject. Thus, prophylactic administration of the vaccine can protect the recipient subject from EBV infection, *e.g.*, to prevent infectious mononucleosis or lymphoproliferative diseases. Therapeutic administration of the vaccine or immunotherapy can protect the recipient from EBV-infection-mediated pathogenesis, *e.g.*, to treat a disease or disorder such as an EBV-associated neoplasm, EBV-associated neoplasms include Hodgkin's lymphoma, endemic Burkitt's lymphoma, nasopharyngeal carcinoma, T cell lymphoma, gastric carcinoma, and uterine leiomyosarcoma.

10 The term "subject" as used herein refers to an animal that supports EBV. In particular, the term refers to a human.

15 The term "vector for expression in humans" as used herein means that the vector at least includes a promoter that is effective in human cells, and preferably that the vector is safe and effective in humans. Such a vector will, for example, omit extraneous genes not involved in developing immunity. If it is a viral vector, it will omit regions that permit replication and development of a robust infection, and will be engineered to avoid development of replication competence *in vivo*. Such vectors are preferably safe for use in humans; in a more preferred embodiment, the vector is approved by a government regulatory agency (such as the Food and Drug Administration) for clinical testing or use in humans. Specific vectors are described in greater detail below.

20 An "adjuvant" is a molecule or composition that potentiates the immune response to an immunogen. An adjuvant is "acceptable for use in a human" when it is pharmaceutically acceptable, as defined below. Examples of adjuvants are provided below.

-12-

The Immuno-protective Antigen of Epstein Barr Virus

The present invention provides an immunoprotective antigen of Epstein Barr Virus, a protective or therapeutic protein or DNA vaccine, and immunotherapy using EBNA-1 charged dendritic cells to prevent or treat EBV infection. The immunoprotective antigen is an immunogenic EBNA-1 polypeptide. As discussed in greater detail below, an EBNA-1 polypeptide can be an EBNA-1 protein, a fusion protein comprising an amino acid sequence, or a fragment of EBNA-1 that includes the immunoprotective epitope.

The term "immunogenic EBNA-1-polypeptide" refers to the EBNA-1 protein, or a portion thereof, that is immunogenic and elicits a protective immune response when administered to an animal. Thus, an EBNA-1 immunoprotective antigen need not be the entire protein. The protective immune response generally involves cellular immunity at the CD4⁺ T cell level.

The immunogenic polypeptide can comprise an immuno-protective EBNA-1 antigen from any strain of Epstein Barr Virus, or sequence variants of EBNA-1, as found in nasopharyngeal carcinoma and infected individuals (Chen *et al.*, J. Gen. Virol., 80:447, 1999; Gutierrez *et al.*, J. Gen. Virol., 78:1663, 1997).

As used herein, the term "immunogenic" means that the polypeptide is capable of eliciting a humoral or cellular immune response, and preferably both. An immunogenic polypeptide is also antigenic. A molecule is "antigenic" when it is capable of specifically interacting with an antigen recognition molecule of the immune system, such as an immunoglobulin (antibody) or T cell antigen receptor. An antigenic polypeptide contains an epitope of at least about five, and preferably at least about 10, amino acids. An antigenic portion of a polypeptide, also called herein the epitope, can be that portion that is immunodominant for antibody or T cell receptor recognition, or it can be a portion used to generate an antibody to the molecule by conjugating the antigenic portion to a carrier polypeptide for immunization. A molecule that is antigenic need not be itself immunogenic, *i.e.*, capable of eliciting an immune response without a carrier.

The term "carrier polypeptide" as used herein refers to a protein or

-13-

immunogenic fragment thereof that can be conjugated or joined with the immunogenic EBNA-1 to enhance immunogenicity of the polypeptide. Examples of carrier proteins include, but are by no means limited to, keyhole limpet hemocyanin (KLY), albumin, cholera toxin (discussed in greater detail below), heat labile enterotoxin (LT), and the like. While chemical cross-linking of a peptide comprising the immuno-protective epitope of EBNA-1 with the carrier polypeptide can be used to prepare an immunogenic polypeptide, preferably the two components are prepared as a chimeric construct for expression as a fusion polypeptide.

In addition, chimeric fusion polypeptides of the immunogenic polypeptide with a purification handle, such as FLAG or GST (for immunopurification), or a HIS-tag (for Ni-chelation purification), are contemplated.

Where the full length recombinant EBNA-1 is used as the immunogenic polypeptide, preferably it is free from viral components, *e.g.*, in distinction to vaccines comprising whole killed or attenuated virus. EBNA-1 polypeptide can be purified after recombinant expression, or it can be delivered by expression *in situ*, *i.e.*, by expression from a vector (a DNA vaccine).

In addition, the present invention permits use of various mutants, sequence conservative variants, and functional conservative variants of EBNA-1, provided that all such variants retain the required immuno-protective effect.

The terms "mutant" and "mutation" mean any detectable change in genetic material, *e.g.* DNA, or any process, mechanism, or result of such a change. This includes gene mutations, in which the structure (*e.g.* DNA sequence) of a gene is altered, any gene or DNA arising from any mutation process, and any expression product (*e.g.* protein) expressed by a modified gene or DNA sequence. The term "variant" may also be used to indicate a modified or altered gene, DNA sequence, enzyme, cell, etc., *i.e.*, any kind of mutant.

"Sequence-conservative variants" of a polynucleotide sequence are those in which a change of one or more nucleotides in a given codon position results in no alteration in the amino acid encoded at that position. Allelic variants can be sequence-conservative variants.

-14-

"Function-conservative variants" are those in which a given amino acid residue in a protein or enzyme has been changed without altering the overall conformation and function of the polypeptide, including, but not limited to, replacement of an amino acid with one having similar properties (such as, for example, polarity, hydrogen bonding potential, acidic, basic, hydrophobic, aromatic, and the like). Some allelic variations result in functional-conservative variants, such that an amino acid substitution does not dramatically affect protein function. Similarly, homologous proteins can be function-conservative variants. Amino acids with similar properties are well known in the art. For example, arginine, histidine and lysine are hydrophilic-basic amino acids and may be interchangeable. Similarly, isoleucine, a hydrophobic amino acid, may be replaced with leucine, methionine or valine. Such changes are expected to have little or no effect on the apparent molecular weight or isoelectric point of the protein or polypeptide. Amino acids other than those indicated as conserved may differ in a protein or enzyme so that the percent protein or amino acid sequence similarity between any two proteins of similar function may vary and may be, for example, from 70% to 99% as determined according to an alignment scheme such as by the Cluster Method, wherein similarity is based on the MEGALIGN algorithm. A "function-conservative variant" also includes a polypeptide or enzyme which has at least 60 % amino acid identity as determined by BLAST or FASTA algorithms, preferably at least 75%, most preferably at least 85%, and even more preferably at least 90%, and which has the same or substantially similar properties or functions as the native or parent protein or enzyme to which it is compared.

As used herein, the term "homologous" in all its grammatical forms and spelling variations refers to the relationship between proteins that possess a "common evolutionary origin," including proteins from superfamilies (*e.g.*, the immunoglobulin superfamily) and homologous proteins from different species (*e.g.*, myosin light chain, etc.) (Reeck, *et al.*, Cell 50:667, 1987). Such proteins (and their encoding genes) have sequence homology, as reflected by their sequence similarity, whether in terms of percent similarity or the presence of specific residues or motifs.

-15-

Accordingly, the term "sequence similarity" in all its grammatical forms refers to the degree of identity or correspondence between nucleic acid or amino acid sequences of proteins that may or may not share a common evolutionary origin (*see* Reeck, *et al.*, *supra*). However, in common usage and in the instant application, the term "homologous," when modified with an adverb such as "highly," may refer to sequence similarity and may or may not relate to a common evolutionary origin.

In a specific embodiment, two DNA sequences are "substantially homologous" or "substantially similar" when a sufficient number of the nucleotides match over the defined length of the DNA sequences to differentiate the sequences from other sequences, as determined by sequence comparison algorithms, such as BLAST, FASTA, DNA Strider, etc. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system.

Similarly, in a particular embodiment, two amino acid sequences are "substantially homologous" or "substantially similar" when enough of the amino acids are identical or similar (functionally identical) over a defined length to differentiate the sequences from other sequences. Preferably, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, *Version 7*, Madison, Wisconsin) pileup program, or any of the programs described above (BLAST, FASTA, etc.).

Furthermore, it should be noted that depending on the expression system employed, the expressed protein can differ from the predicted amino acid sequence encoded by a coding sequence. For example, a construct for expression of the immunogenic polypeptide can express a protein comprising a signal sequence, which may be cleaved or not during cellular processing. In addition, other proteolytic cleavages may occur during expression. If the polypeptide is expressed in eukaryotic cells, it may be glycosylated if it contains a glycosylation site. Other possible changes include N-methylation, and the like.

-16-

As used herein, the term "isolated" means that the referenced material is removed from its native environment, *e.g.*, a cell. Thus, an isolated biological material can be free of some or all cellular components, *i.e.*, components of the cells in which the native material occurs naturally (*e.g.*, cytoplasmic or membrane component). A material shall be deemed isolated if it is present in a cell extract or if it is present in a heterologous cell or cell extract. In the case of nucleic acid molecules, an isolated nucleic acid includes a PCR product, an isolated mRNA, a cDNA, or a restriction fragment. In another embodiment, an isolated nucleic acid is preferably excised from the chromosome in which it may be found, and more preferably is no longer joined or proximal to non-coding regions (but may be joined to its native regulatory regions or portions thereof), or to other genes, located upstream or downstream of the gene contained by the isolated nucleic acid molecule when found in the chromosome. In yet another embodiment, the isolated nucleic acid lacks one or more introns. Isolated nucleic acid molecules include sequences inserted into plasmids, cosmids, artificial chromosomes, and the like, *i.e.*, when it forms part of a chimeric recombinant nucleic acid construct. Thus, in a specific embodiment, a recombinant nucleic acid is an isolated nucleic acid. An isolated protein may be associated with other proteins or nucleic acids, or both, with which it associates in the cell, or with cellular membranes if it is a membrane-associated protein. An isolated organelle, cell, or tissue is removed from the anatomical site in which it is found in an organism. An isolated material may be, but need not be, purified.

The term "purified" as used herein refers to material that has been isolated under conditions that reduce or eliminate the presence of unrelated materials, *i.e.*, contaminants, including native materials from which the material is obtained. For example, a purified protein is preferably substantially free of other proteins or nucleic acids with which it is associated in a cell; a purified nucleic acid molecule is preferably substantially free of proteins or other unrelated nucleic acid molecules with which it can be found within a cell. As used herein, the term "substantially free" is used operationally, in the context of analytical testing of the material. Preferably, purified material substantially free of contaminants is at least 50% pure; more

-17-

preferably, at least 90% pure, and more preferably still at least 99% pure. Purity can be evaluated by chromatography, gel electrophoresis, immunoassay, composition analysis, biological assay, and other methods known in the art.

Methods for purification are well-known in the art. For example,

5 nucleic acids can be purified by precipitation, chromatography (including without limitation preparative solid phase chromatography, oligonucleotide hybridization, and triple helix chromatography), ultracentrifugation, and other means. Polypeptides and proteins can be purified by various methods including, without limitation, preparative disc-gel electrophoresis and isoelectric focusing; affinity, HPLC, reversed-phase

10 HPLC, gel filtration or size exclusion, ion exchange and partition chromatography; precipitation and salting-out chromatography; extraction; and countercurrent distribution. For some purposes, it is preferable to produce the polypeptide in a recombinant system in which the protein contains an additional sequence tag that facilitates purification, such as, but not limited to, a polyhistidine sequence, or a

15 sequence that specifically binds to an antibody, such as FLAG and GST. The polypeptide can then be purified from a crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively, antibodies produced against the protein or against peptides derived therefrom can be used as purification reagents. Cells can be purified by various techniques, including

20 centrifugation, matrix separation (*e.g.*, nylon wool separation), panning and other immunoselection techniques, depletion (*e.g.*, complement depletion of contaminating cells), and cell sorting (*e.g.*, fluorescence activated cell sorting (FACS)). Other purification methods are possible and contemplated herein. A purified material may contain less than about 50%, preferably less than about 75%, and most preferably less

25 than about 90%, of the cellular components, media, proteins, or other undesirable components or impurities (as context requires), with which it was originally associated. The term "substantially pure" indicates the highest degree of purity which can be achieved using conventional purification techniques known in the art.

In a specific embodiment, the term "about" or "approximately" means

30 within 20%, preferably within 10%, and more preferably within 5% of a given value

or range. Alternatively, logarithmic terms used in biology, the term "about" can mean within an order of magnitude of a given value, and preferably within one-half an order of magnitude of the value.

5 **Recombinant Expression Systems**

The present invention contemplates various cloning and expression vectors for expression of the immunogenic polypeptides described herein. Such expression vectors can be used to transform cells *in vitro* to produce immunogenic polypeptides for protein vaccines, or *in vivo* to express the immunogenic polypeptide for a DNA vaccine.

The coding sequence for an immunogenic polypeptide may, and preferably does, include a signal sequence, which can be a heterologous signal sequence, *e.g.*, for optimized signal sequence processing in a bacterial, yeast, insect, or mammalian cell. The term "signal sequence" is used herein to refer to the N-terminal, hydrophobic sequence found on most secreted proteins that identifies it for processing for secretion from the cell. Generally, the signal sequence is cleaved during processing. However, various constructs of the invention can include a partial signal sequence. It is not necessarily the case that the partial signal sequence is processed normally, or that it even provides for translocation during expression, *e.g.*, to the bacterial periplasm.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook *et al.*, 1989"); *DNA Cloning: A Practical Approach*, Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* [B.D. Hames & S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal Cell Culture* [R.I. Freshney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL Press.

(1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

Molecular Biology Definitions

5 A "nucleic acid molecule" (or alternatively "nucleic acid") refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in either
10 single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. This term includes double-stranded DNA found, *inter alia*, in linear (*e.g.*, restriction fragments) or circular DNA molecules, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal
15 convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

A "coding sequence" or a sequence "encoding" an expression product,
20 such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in the production of that RNA, polypeptide, protein, or enzyme, *i.e.*, the nucleotide sequence encodes an amino acid sequence for that polypeptide, protein or enzyme. A coding sequence for a protein may include a start codon (usually ATG) and a stop codon.

25 The coding sequences herein may be flanked by natural regulatory (expression control) sequences, or may be associated with heterologous sequences, including promoters, internal ribosome entry sites (IRES) and other ribosome binding site sequences, enhancers, response elements, suppressors, signal sequences, polyadenylation sequences, introns, 5'- and 3'- non-coding regions, and the like.

30 The term "gene", also called a "structural gene" means a DNA

-20-

sequence that codes for or corresponds to a particular sequence of amino acids which comprise all or part of one or more proteins, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed.

5 The introduced gene or coding sequence may also be called a "cloned", "foreign", or "heterologous" gene or sequence, and may include regulatory or control sequences used by a cell's genetic machinery. The gene or sequence may include nonfunctional sequences or sequences with no known function.

10 The term "host cell" means any cell of any organism that is selected, modified, transformed, grown, or used or manipulated in any way, for the production of a substance by the cell, for example the expression by the cell of a gene, a DNA or RNA sequence, a protein or an enzyme. A host cell has been "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. A cell has been "transformed" by exogenous or heterologous DNA when the
15 transfected DNA is expressed and effects a function or phenotype on the cell in which it is expressed. The term "expression system" means a host cell transformed by a compatible expression vector and cultured under suitable conditions *e.g.* for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell.

20 Proteins and polypeptides can be made in the host cell by expression of recombinant DNA. As used herein, the term "polypeptide" refers to an amino acid-based polymer, which can be encoded by a nucleic acid or prepared synthetically. Polypeptides can be proteins, protein fragments, chimeric proteins, etc. Generally, the term "protein" refers to a polypeptide expressed endogenously in a cell, *e.g.*, the
25 naturally occurring form (or forms) of the amino acid-based polymer. Generally, a DNA sequence having instructions for a particular protein or enzyme is "transcribed" into a corresponding sequence of RNA. The RNA sequence in turn is "translated" into the sequence of amino acids which form the protein or enzyme. An "amino acid sequence" is any chain of two or more amino acids.

30 A "promoter sequence" is a DNA regulatory region capable of binding

-21-

RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control" or "operatively associated with" of transcriptional and translational (*i.e.*, expression) control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced (if it contains introns) and translated into the protein encoded by the coding sequence.

The terms "express" and "expression" mean allowing or causing the information in a gene or DNA sequence to become manifest, for example producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene or DNA sequence. A DNA sequence is expressed in or by a cell to form an "expression product" such as a protein. The expression product itself, *e.g.* the resulting protein, may also be said to be "expressed" by the cell. An expression product can be characterized as intracellular, extracellular, or secreted. The term "intracellular" means something that is inside a cell. The term "extracellular" means something that is outside a cell, either in the cell membrane or secreted from the cell. A substance is "secreted" by a cell if it appears in significant measure in the external medium outside the cell, from somewhere on or inside the cell.

The terms "vector", "cloning vector" and "expression vector" mean the vehicle by which a DNA or RNA sequence (*e.g.*, a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression (*e.g.*, transcription and translation) of the introduced sequence. Vectors include plasmids, phages, viruses, etc. A "cassette" refers to a DNA coding sequence or segment of DNA that codes for an expression product that can be inserted into a vector at defined restriction

-22-

sites. The cassette restriction sites are designed to ensure insertion of the cassette in the proper reading frame. Generally, foreign DNA is inserted at one or more restriction sites of the vector DNA, and then is carried by the vector into a host cell along with the transmissible vector DNA. A segment or sequence of DNA having inserted or added DNA, such as an expression vector, can also be called a "DNA construct." A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic hosts. Non-limiting examples include pKK plasmids (Clontech), pUC plasmids, pET plasmids (Novagen, Inc., Madison, WI), pRSET or pREP plasmids (Invitrogen, San Diego, CA), or pMAL plasmids (New England Biolabs, Beverly, MA), and many appropriate host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. Recombinant cloning vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, *e.g.* antibiotic resistance, and one or more expression cassettes.

The term "expression system" means a host cell and compatible vector under suitable conditions, *e.g.*, for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell. Expression systems include bacterial, insect, or mammalian host cells and vectors. Bacterial and insect cell expression is exemplified *infra*. Suitable mammalian cells include C12 cells, CHO cells, HeLa cells, 293 and 293T (human kidney cells), COS cells, mouse primary myoblasts, and NIH 3T3 cells.

The term "heterologous" refers to a combination of elements not naturally occurring. For example, heterologous DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. Preferably, the heterologous DNA includes a gene foreign to the cell. A heterologous expression regulatory element is a such an element operatively associated with a different gene than the one it is operatively associated with in nature. In the context of the present invention, an gene is heterologous to the recombinant vector DNA in which it is inserted for cloning or expression, and it is heterologous to a host cell containing such a vector, in

-23-

which it is expressed, *e.g.*, a CHO cell.

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (*see* Sambrook, *et al.*, *supra*). For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (*see* Sambrook, *et al.*, *supra*, 9.50-9.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (*see* Sambrook, *et al.*, *supra*, 11.7-11.8). A minimum length for a hybridizable nucleic acid is at least about 10 nucleotides; preferably at least about 15 nucleotides; and more preferably the length is at least about 20 nucleotides.

Expression Vectors

A wide variety of host/expression vector combinations (*i.e.*, expression systems) may be employed in expressing the immunogenic polypeptides of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, *e.g.*, *E. coli* plasmids col El, pCR1, pBR322, SV40 and pMal-C2, pET, pGEX (Smith, *et al.*, Gene 67:31-40, 1988), pMB9 and their derivatives, plasmids such as RP4; gram positive vectors such as *Strep. gordonii*; phage DNAs, *e.g.*, the numerous derivatives of phage λ , *e.g.*, NM989, and other phage DNA, *e.g.*, M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2 μ plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

Expression of the protein or polypeptide may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. Promoters which may be used to

-24-

control gene expression include, but are not limited to, cytomegalovirus (CMV) promoter (U.S. Patent Nos. 5,385,839 and 5,168,062), the SV40 early promoter region (Benoist and Chambon, *Nature* 290:304-310, 1981), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, *Cell* 22:787-797, 1980), the herpes thymidine kinase promoter (Wagner, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 78:1441-1445, 1981), the regulatory sequences of the metallothionein gene (Brinster, *et al.*, *Nature* 296:39-42, 1982); prokaryotic expression vectors such as the b-lactamase promoter (Villa-Komaroff, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 75:3727-3731, 1978), or the *tac* promoter (DeBoer, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 80:21-25, 1983); see also "Useful proteins from recombinant bacteria" in *Scientific American*, 242:74-94, 1980; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and control regions that exhibit hematopoietic tissue specificity, in particular: immunoglobulin gene control region, which is active in lymphoid cells (Grosschedl *et al.*, *Cell*, 38:647, 1984; Adames *et al.*, *Nature*, 318:533, 1985; Alexander *et al.*, *Mol. Cell Biol.*, 7:1436, 1987); beta-globin gene control region which is active in myeloid cells (Mogran, *et al.*, *Nature* 315:338-340, 1985; Kollias, *et al.*, *Cell* 46:89-94, 1986), hematopoietic stem cell differentiation factor promoters; erythropoietin receptor promoter (Maouche, *et al.*, *Blood*, 15:2557, 1991), etc; and control regions that exhibit mucosal epithelial cell specificity.

Preferred vectors, particularly for cellular assays *in vitro* and vaccination *in vivo* or *ex vivo*, are viral vectors, such as lentiviruses, retroviruses, herpes viruses, adenoviruses, adeno-associated viruses, vaccinia viruses, baculoviruses, Fowl pox, AV-pox, modified vaccinia Ankara (MVA) and other recombinant viruses with desirable cellular tropism. In a specific embodiment, a vaccinia virus vector is used to infect dendritic cells. In another specific embodiment, a baculovirus vector that expresses EBNA-1 is prepared. Thus, a vector encoding an immunogenic polypeptide can be introduced *in vivo*, *ex vivo*, or *in vitro* using a viral vector or through direct introduction of DNA. Expression in targeted tissues can be

-25-

effected by targeting the transgenic vector to specific cells, such as with a viral vector or a receptor ligand, or by using a tissue-specific promoter, or both. Targeted gene delivery is described in International Patent Publication WO 95/28494, published October 1995.

5 Viral vectors commonly used for *in vivo* or *ex vivo* targeting and vaccination procedures are DNA-based vectors and retroviral vectors. Methods for constructing and using viral vectors are known in the art (*see, e.g.*, Miller and Rosman, BioTechniques, 7:980-990, 1992). Preferably, the viral vectors are replication defective, that is, they are unable to replicate autonomously in the target
10 cell. Preferably, the replication defective virus is a minimal virus, *i.e.*, it retains only the sequences of its genome which are necessary for encapsidating the genome to produce viral particles.

 DNA viral vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus
15 (EBV), adenovirus, adeno-associated virus (AAV), vaccinia virus, and the like. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector (Kapliitt, *et al.*, Molec. Cell. Neurosci. 2:320-330, 1991; International Patent Publication No. WO 94/21807, published September 29, 1994; International Patent Publication No. WO 92/05263, published April 2, 1994); an attenuated
20 adenovirus vector, such as the vector described by Stratford-Perricaudet, *et al.* (J. Clin. Invest. 90:626-630, 1992; see also La Salle, *et al.*, Science 259:988-990, 1993); and a defective adeno-associated virus vector (Samulski, *et al.*, J. Virol. 61:3096-3101, 1987; Samulski, *et al.*, J. Virol. 63:3822-3828, 1989; Lebkowski, *et al.*, Mol. Cell. Biol. 8:3988-3996, 1988).

25 Various companies produce viral vectors commercially, including but by no means limited to Avigen, Inc. (Alameda, CA; AAV vectors), Cell Genesys (Foster City, CA; retroviral, adenoviral, AAV vectors, and lentiviral vectors), Clontech (retroviral and baculoviral vectors), Genovo, Inc. (Sharon Hill, PA; adenoviral and AAV vectors), Genvec (adenoviral vectors), IntroGene (Leiden,
30 Netherlands; adenoviral vectors), Molecular Medicine (retroviral, adenoviral, AAV,

-26-

and herpes viral vectors), Norgen (adenoviral vectors), Oxford BioMedica (Oxford, United Kingdom; lentiviral vectors), and Transgene (Strasbourg, France; adenoviral, vaccinia, retroviral, and lentiviral vectors).

Adenovirus vectors. Adenoviruses are eukaryotic DNA viruses that can be modified to efficiently deliver a nucleic acid of the invention to a variety of cell types. Various serotypes of adenovirus exist. Of these serotypes, preference is given, within the scope of the present invention, to using type 2 or type 5 human adenoviruses (Ad 2 or Ad 5) or adenoviruses of animal origin (see WO94/26914). Those adenoviruses of animal origin which can be used within the scope of the present invention include adenoviruses of canine, bovine, murine (example: Mav1, Beard, *et al.*, Virology 75 (1990) 81), ovine, porcine, avian, and simian (example: SAV) origin. Preferably, the adenovirus of animal origin is a canine adenovirus, more preferably a CAV2 adenovirus (e.g. Manhattan or A26/61 strain (ATCC VR-800), for example). Various replication defective adenovirus and minimum adenovirus vectors have been described (WO94/26914, WO95/02697, WO94/28938, WO94/28152, WO94/12649, WO95/02697 WO96/22378). The replication defective recombinant adenoviruses according to the invention can be prepared by any technique known to the person skilled in the art (Levero, *et al.*, Gene 101:195 1991; EP 185 573; Graham, EMBO J. 3:2917, 1984; Graham, *et al.*, J. Gen. Virol. 36:59 1977). Recombinant adenovirus is an efficient and non-perturbing vector for human dendritic cells (Zhong *et al.*, Eur. J. Immunol., 29:964, 1999; DiNicola *et al.*, Cancer Gene Ther., 5:350-6, 1998). Recombinant adenoviruses are recovered and purified using standard molecular biological techniques, which are well known to one of ordinary skill in the art.

Adeno-associated viruses. The adeno-associated viruses (AAV) are DNA viruses of relatively small size which can integrate, in a stable and site-specific manner, into the genome of the cells which they infect. They are able to infect a wide spectrum of cells without inducing any effects on cellular growth, morphology or differentiation, and they do not appear to be involved in human pathologies. The AAV genome has been cloned, sequenced and characterized. The use of vectors

-27-

derived from the AAVs for transferring genes *in vitro* and *in vivo* has been described (see WO 91/18088; WO 93/09239; US 4,797,368, US 5,139,941, EP 488 528). The replication defective recombinant AAVs according to the invention can be prepared by cotransfecting a plasmid containing the nucleic acid sequence of interest flanked by two AAV inverted terminal repeat (ITR) regions, and a plasmid carrying the AAV encapsidation genes (rep and cap genes), into a cell line which is infected with a human helper virus (for example an adenovirus). The AAV recombinants which are produced are then purified by standard techniques. These viral vectors are also effective for gene transfer into human dendritic cells (DiNicola *et al.*, *supra*).

Retrovirus vectors. In another embodiment the gene can be introduced in a retroviral vector, *e.g.*, as described in Anderson, *et al.*, U.S. Patent No. 5,399,346; Mann, *et al.*, 1983, Cell 33:153; Temin, *et al.*, U.S. Patent No. 4,650,764; Temin, *et al.*, U.S. Patent No. 4,980,289; Markowitz, *et al.*, 1988, J. Virol. 62:1120; Temin, *et al.*, U.S. Patent No. 5,124,263; EP 453242, EP178220; Bernstein, *et al.* Genet. Eng. 7 (1985) 235; McCormick, BioTechnology 3 (1985) 689; International Patent Publication No. WO 95/07358, published March 16, 1995, by Dougherty, *et al.*; and Kuo, *et al.*, 1993, Blood 82:845. The retroviruses are integrating viruses which infect dividing cells. The retrovirus genome includes two LTRs, an encapsidation sequence and three coding regions (gag, pol and env). In recombinant retroviral vectors, the gag, pol and env genes are generally deleted, in whole or in part, and replaced with a heterologous nucleic acid sequence of interest. These vectors can be constructed from different types of retrovirus, such as, HIV, MoMuLV ("murine Moloney leukaemia virus" MSV ("murine Moloney sarcoma virus"), HaSV ("Harvey sarcoma virus"); SNV ("spleen necrosis virus"); RSV ("Rous sarcoma virus") and Friend virus. Suitable packaging cell lines have been described in the prior art, in particular the cell line PA317 (US 4,861,719); the PsiCRIP cell line (WO 90/02806) and the GP+envAm-12 cell line (WO 89/07150). In addition, the recombinant retroviral vectors can contain modifications within the LTRs for suppressing transcriptional activity as well as extensive encapsidation sequences which may include a part of the gag gene (Bender, *et al.*, J. Virol. 61:1639, 1987). Recombinant retroviral vectors are

-28-

purified by standard techniques known to those having ordinary skill in the art.

Retrovirus vectors can also be introduced by DNA viruses, which permits one cycle of retroviral replication and amplifies transfection efficiency (see WO 95/22617, WO 95/26411, WO 96/39036, WO 97/19182).

- 5 **Lentivirus vectors.** In another embodiment, lentiviral vectors are can be used as agents for the direct delivery and sustained expression of a transgene in several tissue types, including brain, retina, muscle, liver and blood. The vectors can efficiently transduce dividing and nondividing cells in these tissues, and maintain long-term expression of the gene of interest. For a review, *see*, Naldini, Curr. Opin. Biotechnol., 9:457-63, 1998; *see also* Zufferey, *et al.*, J. Virol., 72:9873-80, 1998).
10 Lentiviral packaging cell lines are available and known generally in the art. They facilitate the production of high-titer lentivirus vectors for gene therapy. An example is a tetracycline-inducible VSV-G pseudotyped lentivirus packaging cell line which can generate virus particles at titers greater than 106 IU/ml for at least 3 to 4 days
15 (Kafri, *et al.*, J. Virol., 73: 576-584, 1999). The vector produced by the inducible cell line can be concentrated as needed for efficiently transducing nondividing cells *in vitro* and *in vivo*.

- Vaccinia virus vectors.** Vaccinia virus is a member of the pox virus family and is characterized by its large size and complexity. Vaccinia virus DNA is
20 double-stranded and terminally crosslinked so that a single stranded circle is formed upon denaturation of the DNA. The virus has been used for approximately 200 years in a vaccine against smallpox and the properties of the virus when used in a vaccine are known (Paoletti, Proc. Natl. Acad. Sci. U.S.A., 93:11349-53, 1996; and Ellner, Infection, 26:263-9,1998). The risks of vaccination with vaccinia virus are well
25 known and well defined and the virus is considered relatively benign. Vaccinia virus vectors can be used for the insertion and expression of foreign genes. The basic technique of inserting foreign genes into the vaccinia vector and creating synthetic recombinants of the vaccinia virus has been described (see U.S. Pat. No. 4,603,112, U.S. Pat. No. 4,722,848, U.S. Pat. No.4, 769, 330 and U.S. Pat. No. 5,364,773). A
30 large number of foreign (*i.e.* non-vaccinia) genes have been expressed in vaccinia,

often resulting in protective immunity (reviewed by Yamanouchi, Barrett, and Kai, Rev. Sci. Tech., 17:641-53, 1998; Yokoyama, *et al.*, J. Vet. Med. Sci., 59:311-22, 1997; and see Osterhaus, *et al.*, Vaccine, 16:1479-81 1998; and Gherardi *et al.*, J. Immunol., 162:6724-33, 1999). Vaccinia virus may be inappropriate for

administration to immunocompromised or immunosuppressed individuals.

Alternative pox viruses which may be used in the invention include Fowl pox, AV-pox, and modified vaccinia Ankara (MVA) virus. The preferred embodiment to improve the immunogenic potential of these alternative viruses is to deliver the viruses containing EBNA-1 directly to dendritic cells, and then induce the dendritic cells to mature.

Nonviral vectors. In another embodiment, the vector can be introduced *in vivo* by lipofection, as naked DNA, or with other transfection facilitating agents (peptides, polymers, etc.). Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker (Felgner, *et al.*, Proc. Natl. Acad. Sci. U.S.A. 84:7413-7417, 1987; Felgner and Ringold, Science 337:387-388, 1989; see Mackey, *et al.*, Proc. Natl. Acad. Sci. U.S.A. 85:8027-8031, 1988; Ulmer, *et al.*, Science 259:1745-1748, 1993). Useful lipid compounds and compositions for transfer of nucleic acids are described in International Patent Publications WO95/18863 and WO96/17823, and in U.S. Patent No. 5,459,127.

Lipids may be chemically coupled to other molecules for the purpose of targeting (see Mackey, *et al.*, *supra*). Targeted peptides, *e.g.*, hormones or neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to liposomes chemically.

Other molecules are also useful for facilitating transfection of a nucleic acid *in vivo*, such as a cationic oligopeptide (*e.g.*, International Patent Publication WO95/21931), peptides derived from DNA binding proteins (*e.g.*, International Patent Publication WO96/25508), or a cationic polymer (*e.g.*, International Patent Publication WO95/21931).

Alternatively, non-viral DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, *e.g.*,

-30-

electroporation, microinjection, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun (ballistic transfection; see, *e.g.*, U.S. Pat. No. 5,204,253, U.S. Pat. No. 5,853,663, U.S. Pat. No. 5,885,795, and U.S. Pat. No. 5,702,384 and see Sanford, TIB-TECH, 6:299-302, 1988; Fynan *et al.*, Proc. Natl. Acad. Sci. U.S.A., 90:11478-11482, 1993; and Yang *et al.*, Proc. Natl. Acad. Sci. U.S.A., 87:1568-9572, 1990), or use of a DNA vector transporter (see, *e.g.*, Wu, *et al.*, J. Biol. Chem. 267:963-967, 1992; Wu and Wu, J. Biol. Chem. 263:14621-14624, 1988; Hartmut, *et al.*, Canadian Patent Application No. 2,012,311, filed March 15, 1990; Williams, *et al.*, Proc. Natl. Acad. Sci. USA 88:2726-2730, 1991). Receptor-mediated DNA delivery approaches can also be used (Curiel, *et al.*, Hum. Gene Ther. 3:147-154, 1992; Wu and Wu, J. Biol. Chem. 262:4429-4432, 1987). US Patent Nos. 5,580,859 and 5,589,466 disclose delivery of exogenous DNA sequences, free of transfection facilitating agents, in a mammal. Recently, a relatively low voltage, high efficiency *in vivo* DNA transfer technique, termed electrotransfer, has been described (Mir, *et al.*, C.P. Acad. Sci., 321:893, 1998; WO 99/01157; WO 99/01158; WO 99/01175).

Vaccine Technology and Immunotherapy

As noted above, the present invention contemplates polypeptide vaccines, and DNA vaccines to deliver an immunogenic EBNA-1 polypeptide to prevent or treat an Epstein Barr Virus infection, or an associated disease (*e.g.*, infectious mononucleosis, endemic Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma, T cell lymphoma, gastric carcinoma, and uterine leiomyosarcoma, and possibly chronic diseases such as chronic fatigue syndrome).

The vaccines of the invention are broadly applicable to protect an animal from infection by Epstein Barr Virus. The term "protect" is used herein to mean for the treatment or prevention of Epstein Barr Virus infection. Thus, any animal susceptible to this type of infection can be vaccinated. EBV shows a greater similarity phylogenetically to gammaherpesviruses, herpesvirus saimiri, and bovine herpesvirus 4 than to other classes of herpesvirus (Karlin, *et al.*, J. Virol.,

68:1886;902, 1994; and Bublot, *et al.*, Virology, 190:654-65, 1992). Animal models for EBV occur in some species of New World monkeys (Franken, *et al.*, J. Virol., 69:8011-9, 1995) as well as in mice (Mistrikova and Mrmusova, Acta. Virol., 42:79-82, 1998, Weck, *et al.*, J. Virol., 73:4651-61, 1999; and Simas and Efstathiou, Trends Microbiol., 6:276-82, 1998) and rabbits (Wutzler, *et al.*, Arch. Virol., 140:1979-95, 1995; and Handley, *et al.*, Vet. Microbiol., 47:167-81, 1995). At least one EBV-like herpesvirus that infects monkeys contains a gene with homology to EBNA-1 (Li, *et al.*, Int. J. Cancer, 59:287-95, 1994). Animals infected with EBV-like viruses could be treated with a vaccine of the invention to prevent or treat disease.

Polypeptide Vaccines

As used herein, the term "polypeptide vaccine" refers to a vaccine comprising an immunogenic polypeptide and, generally, an adjuvant. The term "adjuvant" refers to a compound or mixture that enhances the immune response to an antigen. An adjuvant can serve as a tissue depot that slowly releases the antigen and also as a lymphoid system activator that non-specifically enhances the immune response (Hood, *et al.*, *Immunology, Second Ed.*, 1984, Benjamin/Cummings: Menlo Park, California, p. 384). Often, a primary challenge with an antigen alone, in the absence of an adjuvant, will fail to elicit a humoral or cellular immune response. Adjuvants include, but are not limited to, complete Freund's adjuvant, incomplete Freund's adjuvant, saponin, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*. An example of a preferred synthetic adjuvant is QS-21. Alternatively, or in addition, immunostimulatory proteins, as described below, can be provided as an adjuvant or to increase the immune response to a vaccine. Preferably, the adjuvant is pharmaceutically acceptable.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like,

-32-

when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Sterile water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

Certain adjuvants mentioned above, particularly mineral oils and adjuvants containing mineral oils (e.g., Freund's adjuvant) are not acceptable for use in humans.

"DNA" Vaccines

The term "DNA vaccines" is an informal term of art, and is used herein to refer to vaccines delivered by means of a recombinant vector. An alternative, and more descriptive term used herein is "vector vaccine" (since some potential vectors, such as retroviruses and lentiviruses are RNA viruses, and since in some instances non-viral RNA instead of DNA can be delivered to cells). Generally, the vector is administered *in vivo*, but *ex vivo* transduction of appropriate antigen presenting cells, such as dendritic cells, with administration of the transduced cells *in vivo*, is also contemplated. The vector systems described above are ideal for delivery of a vector for expression of an immunogenic polypeptide of the invention.

Vaccination and Immunotherapy Strategies

Various strategies can be employed to vaccinate subjects against *Epstein Barr Virus* infection. The polypeptide vaccine formulations can be delivered by subcutaneous (s.c.), intraperitoneal (i.p.), intramuscular (i.m.), subdermal (s.d.), intradermal (i.d.), or by administration to antigen presenting cells *ex vivo* followed by administration of the cells to the subject. Prior to administration to the subject, the antigen presenting cells may be induced to mature.

Similarly, any of the gene delivery methods described above can be

-33-

used to administer a vector vaccine to a subject, such as naked DNA and RNA delivery, *e.g.*, by gene gun or direct injection.

Vaccination effectiveness may be enhanced by co-administration of an immunostimulatory molecule (Salgaller and Lodge, J. Surg. Oncol., 68:122, 1998), such as an immunostimulatory, immunopotentiating, or pro-inflammatory cytokine, lymphokine, or chemokine with the vaccine, particularly with a vector vaccine. For example, cytokines or cytokine genes such as interleukin (IL)-1, IL-2, IL-3, IL-4, IL-12, IL-13, granulocyte-macrophage (GM)-colony stimulating factor (CSF) and other colony stimulating factors, macrophage inflammatory factor, Flt3 ligand (Lyman, Curr. Opin. Hematol., 5:192, 1998), as well as some key costimulatory molecules or their genes (*e.g.*, B7.1, B7.2) can be used. These immunostimulatory molecules can be delivered systemically or locally as proteins or by expression of a vector that codes for expression of the molecule. The techniques described above for delivery of the immunogenic polypeptide can also be employed for the immunostimulatory molecules.

Dendritic Cell Targeting. Vaccination and particularly immunotherapy may be accomplished through the targeting of dendritic cells (Steinman, J. Lab. Clin. Med., 128:531, 1996; Steinman, Exp. Hematol., 24:859, 1996; Taite *et al.*, Leukemia, 13:653, 1999; Avigan, Blood Rev., 13:51, 1999; DiNicola *et al.*, Cytokines Cell. Mol. Ther., 4:265, 1998). Dendritic cells play a crucial role in the activation of T-cell dependent immunity. Proliferating dendritic cells can be used to capture protein antigens in an immunogenic form *in situ* and then present these antigens in a form that can be recognized by and stimulates T cells (see, *e.g.*, Steinman, Exper. Hematol. 24:859-862, 1996; Inaba, *et al.*, J. Exp. Med., 188:2163-73, 1998 and U.S. Pat. No. 5,851,756). For *ex vivo* stimulation, dendritic cells are plated in culture dishes and exposed to (pulsed with) antigen in a sufficient amount and for a sufficient period of time to allow the antigen to bind to the dendritic cells. Additionally, dendritic cells may be transfected with DNA using a variety of physical or chemical as described by Zhong *et al.*, Eur. J. Immunol., 29:964-72, 1999; Van Tendeloo, *et al.*, Gene Ther., 5:700-7, 1998; Diebold *et al.*,

-34-

Hum. Gene Ther., 10:775-86, 1999; Francotte and Urbain, Proc. Natl. Acad. Sci. USA, 82:8149, 1985 and U.S. Pat. No. 5,891,432 (Casares *et al.*, J. Exp. Med., 186:1481-6, 1997). The pulsed cells can then be transplanted back to the subject undergoing treatment, *e.g.*, by intravenous injection. Preferably autologous dendritic cells, *i.e.*, dendritic cells obtained from the subject undergoing treatment, are used, although it may be possible to use MHC-Class II-matched dendritic cells, which may be obtained from a type-matched donor or by genetic engineering of dendritic cells to express the desired MHC molecules (and preferably suppress expression of undesirable MHC molecules.)

Preferably, the dendritic cells are specifically targeted *in vivo* for expression of EBNA-1. Various strategies are available for targeting dendritic cells *in vivo* by taking advantage of receptors that mediate antigen presentation, such as DEC-205 (Swiggard *et al.*, Cell. Immunol., 165:302-11, 1995; Steinman, Exp. Hematol., 24:859, 1996) and Fc receptors. Targeted viral vectors, discussed above, can also be used. Additionally, dendritic cells may be induced to mature *in vitro* after infection by the viral vector, prior to transplantation *in vivo*.

Mucosal Vaccination. Mucosal vaccine strategies are particularly effective for many pathogenic viruses, since infection often occurs via the mucosa. Additionally, mucosal delivery of recombinant vaccinia virus vaccines may be able to overcome a pre-existing immunity to poxviruses due to previous smallpox vaccination (Belyakov, *et al.*, Proc. Natl. Acad. Sci. U.S.A., 96:4512-7, 1999). The mucosa harbors dendritic cells, which are important targets for EBNA-1 vaccines and immunotherapy. Thus, mucosal vaccination strategies for both polypeptide and DNA vaccines are contemplated. While the mucosa can be targeted by local delivery of a vaccine, various strategies have been employed to deliver immunogenic proteins to the mucosa (these strategies include delivery of DNA vaccines as well, *e.g.*, by using the specific mucosal targeting proteins as vector targeting proteins, or by delivering the vaccine vector in an admixture with the mucosal targeting protein).

For example, in a specific embodiment, the immunogenic polypeptide or vector vaccine can be administered in an admixture with, or as a conjugate or

-35-

chimeric fusion protein with, cholera toxin, such as cholera toxin B or a cholera toxin A/B chimera (Hajishengallis, J Immunol., 154:4322-32, 1995; Jobling and Holmes, Infect Immun., 60:4915-24, 1992). Mucosal vaccines based on use of the cholera toxin B subunit have been described (Lebens and Holmgren, Dev Biol Stand 82:215-27, 1994). In another embodiment, an admixture with heat labile enterotoxin (LT) can be prepared for mucosal vaccination.

Other mucosal immunization strategies include encapsulating the immunogen in microcapsules (U.S. Patents No. 5,075,109, No. 5,820,883, and No. 5,853,763) and using an immunopotentiating membranous carrier (WO 98/0558). Immunogenicity of orally administered immunogens can be enhanced by using red blood cells (rbc) or rbc ghosts (U.S. Patent No. 5,643,577), or by using blue tongue antigen (U.S. Patent No. 5,690,938). Systemic administration of a targeted immunogen can also produce mucosal immunization (see, U.S. Patent No. 5,518,725).

Various strategies can be used to deliver genes for expression in mucosal tissues, such as using chimeric rhinoviruses (U.S. Patent No. 5,714,374), adenoviruses, vaccinia viruses, or specific targeting of a nucleic acid (WO 97/05267).

EXAMPLES

The present invention will be better understood by reference to the following examples, which are provided by way of exemplification and are not intended to limit the invention.

EXAMPLE 1: Identification of positive CD4⁺ response to individual latent EBV products

25

For EBNA-1, we delivered the antigen either exogenously as recombinant protein (Zhang, *et al.*, Nucleic Acids Res., 26:631-7, 1998; Frappier and O'Donnell, J. Biol. Chem., 266:7819-26, 1991), or endogenously via recombinant vaccinia virus constructs. We have uncovered a strong CD4⁺ T cell response to EBNA-1 presented by either pathway, as monitored by T cell activation and proliferation, IFN γ secretion, and CTL activity. This immune response may be

30

-36-

harnessed to resist EBV infection and EBV-associated malignancy.

Material and Methods

Cell lines. The EBV transformed B cell lines LRM (HLA-A2, -B44, -

- 5 DRB1*0401, -DQA1*03, -DQB1*0301, -DP4) (Friede, *et al.*, Biochim. Biophys. Acta., 1316:85-101, 1996) and LG2 (HLA-DRB1*0101, -DQA*0101, -DQB1*0501, -DPA1*0101, -DPB1*0201) (Gorga, *et al.*, J. Biol. Chem., 26:16087-94, 1987) were used. B-LCL were cultured in RPMI-1640 + 10 % FCS + 5 mM glutamine + 20 μ g/ml gentamicin. LCL-BM (HLA-A1, -A3, -B7, -B8, -Cw6, -Cw7, -DR4, -DRw14, 10 -DRw52, -DRw53, -DQw3) and LCL-DC (HLA-A2, -A24, -B38, -B46, -Cw1, -Cw7, -DRB1*1502, -DRB1*0901, -DRB4*01, -DRB5*0101, -DQB1*0502, -DQB1*0303) were generated by culturing PBMC of typed healthy donors with supernatant of the marmoset cell line B95.8 in RPMI-1640 + 20 % FCS + 5 mM glutamine + 20 μ g/ml gentamicin + 1 μ g/ml cyclosporin A. The BSC40 monkey kidney cell line was grown 15 in DMEM + 5 % FCS + 5 mM glutamine + 20 μ g/ml gentamicin and used in plaque assays to titer recombinant VV stocks.

Dendritic cell (DC) and PBMC preparations. Leucocyte

- concentrates (buffy coats) from the New York Blood Center, as well as whole blood from lab donors served as sources of PBMC, isolated by density gradient 20 centrifugation on Ficoll-Paque (Pharmacia). CD2⁺ PBMC were separated by rosetting with neuraminidase (Calbiochem) treated sheep red blood cells (Colorado Serum Company) followed by red cell lysis with 1.66 % ammonium chloride. Where indicated, CD8⁺ or CD4⁺ T cells were depleted with Leu2a or OKT8 (for CD8) or HP2/6 (for CD4) antibodies, followed by incubation with sheep- α -mouse-IgG 25 Dynabeads and a magnetic particle concentrator MCP-1 (Dynal, Norway). DCs were generated from CD2⁺ PBMC as described (Bender, *et al.*, J. Immunol. Methods, 196:121-35, 1996; Romani, *et al.*, J. Immunol. Methods, 196:137-51, 1996). 10⁶ CD2⁺ PBMC / ml were plated in 6-well plates with RPMI-1640 + 1 % single donor plasma + 1000 U/ml rhIL-4 + 1000 U/ml rhGM-CSF + 5 mM glutamine + 20 μ g/ml 30 gentamicin. 100 μ l were replaced with 200 μ l/ml RPMI-1640 + 1 % single donor

-37-

plasma + 5 mM glutamine + 20 µg/ml gentamicin and 1000 U/ml rhIL-4 as well as 1000 U/ml rhGM-CSF were added at day 2, 4 and 6. On day 7, the floating immature DCs were transferred to new plates at 3×10^5 cells / ml and half of the medium was replaced by monocyte conditioned medium to mature the DCs for two days.

5 ***Vaccinia virus stock generation and infection of DCs.*** Recombinant vaccinia viruses were expanded in rabbit kidney RK13 cells. Mature DCs were infected at a MOI of 2 for 1 hour at 37°C and washed three times. The efficiency of infection was checked after 6 to 12 hours by FACS as described using intracellular staining of a vaccinia early protein of 29 kD with the VV1-6B6 antibody.

10 ***Generation of CD4⁺ T cell lines and clones.*** CD8⁺CD2⁺ PBMC were stimulated with mature DCs at a ratio of 30:1 (T:DC). For the CM171198 cell line T cells of the healthy donor CM (HLA-A*0201, -A*6801, -B*4402, -B*0702, -C*0501, -C*0702, -DRB1*1501, -DRB1*0401, -DRB5*01, -DRB4*01, -DQB1*0602, -DQB1*0301) were stimulated for 4 weeks with vvEBNA-1ΔGA infected autologous mature DCs with weekly restimulations including autologous CD2⁺ PBMC as feeders and then alternating with the EBV transformed HLA-DR matched cell line LRM or vvEBNA-1ΔGA infected DCs. The CM110199 cell line was generated from T cells of the donor CM by using DCs that had been incubated with 10 µM of recombinant EBNA-1 protein, added at day 7 together with the maturation stimulus. Purified rEBNA-1 from *E. coli* and baculovirus / insect cell expression systems were alternatively used (Zhang, *et al.*, *supra*, 1998; Frappier and O'Donnell, *supra*). Where indicated, *E. coli* derived DNA-C was used as a control protein (*E. coli* control). After 14 days of stimulation in DMEM + 5 % HS, 50 U/ml rIL-2 was supplemented. For stimulations with the autologous B-LCL, CD2⁺ PBMC of the healthy donor JT were stimulated for 14 days with the irradiated autologous LCL-JT at a B cell to T cell ratio of 1:10 in DMEM + 5 % human serum + 10 U/ml IL-2 (Lymphocult, Dreieich, Germany). Where indicated, CD4⁺ or CD8⁺ T cells were depleted.

25
30 EBNA-1 specific CD4⁺ CTL were cloned under limiting dilution conditions by stimulating 10^5 CD8⁺CD2⁺PBMC with 10^4 autologous B-LCL from

-38-

leukocyte concentrates in 96 well plates for 14 days with one restimulation at day 7. IL-2 was added to the cultures during the restimulation to a final concentration of 10U/ml (Lymphocult). Microcultures were tested in split well ^{51}Cr release assays against autologous DCs infected with vvEBNA-1ΔGA or vvTK⁻, autologous B-LCL or LCL721.221. At this initial T cell number, <30% of the wells developed CTL indicating >90% probability for clonality of the responders (Taswell, *et al.*, J. Exp. Med., 151:1372-85, 1980).

FACS analysis of stimulated CD4⁺ T cell populations and PBMC.

Mature DCs were infected with recombinant VV at an MOI of 2, or with influenza virus (PR8, Puerto Rico/8/34, Spafas Inc., Storrs, CT) at a MOI of 0.5 for 1 hour at 37°C in RPMI-1640 + 5 % HS. DCs were washed twice and 3×10^3 added to 10^5 CD8⁺ CD2⁺ PBMC in 96 well plates for 7 days. At day 7, the cultures were restimulated with 10^5 PBMC and 3×10^3 DCs per well and incubated for another 7 days. The cultures were restimulated with 10^5 irradiated (3000 rad) PBMC and 3×10^3 DCs per well and incubated for another 7 days. After 14 days, cultures were stained for 30 minutes on ice with 1 μl Simultest CD4-FITC/CD8-PE (Becton Dickinson) in 100 μl PBS + 1 % FCS + 0.005 % sodium azide. After 3 washes, cultures were analyzed on a FACScan (Becton Dickinson). CD56 antibody staining (PharMingen) used PE - goat αmouse-IgG antibody (Biosource) as secondary. PBMC were typed for HLA-DR4 using HLA-DR4 antibody (Accurate) as primary and FITC-goat α-mouse IgG antibody (Biosource) as secondary.

ELISPOT assay for IFNγ secreting cells. MAHA S45 plates

(Millipore) were coated with 10 μg/ml αIFNγ antibody 1-D1K (MABTECH) in 50 μl/well 50 mM Na₂CO₃, pH 9.5, overnight at 4°C. Plates were washed 4 times with PBS and blocked with DMEM + 5 % HS for 1 hour at 37°C. Afterwards, 10^5 responder T cells and $3 \times 10^3 - 10^4$ stimulator DCs were added per well and incubated for 1 - 2 days at 37°C. Then the plates were washed 4 times with PBS + 0.05 % Tween 20 and incubated for 2 hours at 37°C with 1 μg/ml biotinylated αIFNγ antibody 7-B6-1 (MABTECH) in 50 μl/well PBS. Afterwards the plates were again washed for 4 times with PBS + 0.1 % Tween 20 and incubated with 20 minutes at

-39-

room temperature preassembled avidin-peroxidase-complexes Vectastain ABC kit (Vector laboratories) for 1 hour at room temperature. After another 4 washes with PBS + 0.1 % Tween 20, 50 μ l/well of stable DAB (Research Genetics) was added for 5 minutes at room temperature. Plates were washed 3 times with water and airdried.

5 SFC / 10^5 cells were counted using a stereomicroscope (mean counts of triplets). Where indicated, α HLA-DR antibody L243 (Lampson, and Levy, J. Immunol., 125:293-9, 1980) or α HLA-A, B, C antibody B-IH9 (Biosource) was added to 5 μ g/ml.

Proliferation assays. Responder T cells (10^5) were incubated with

10 $3 \times 10^3 - 10^4$ stimulator DCs for 5 days in DMEM + 5 % HS. 1 μ Ci 3 H-Thymidine was added / well overnight and harvested by an automatic device (Skatron) and counted in a Betaplate 1205 (LKB Wallac). Counts represent mean values of triplicates.

^{51}Cr release assay. Targets were labeled with 50 μ Ci $\text{Na}_2^{51}\text{CrO}_4$ for 45 minutes at 37°C . Labeled targets were incubated for 4 hours with CTL in RPMI +

15 10% FCS + 2 mM glutamine. The supernatant was harvested using a Skatron harvesting system and radioactivity measured in a γ counter (1470 Wizard, Wallac, Turku, Finland). Percent specific lysis was $([\text{cpm experimental well} - \text{cpm spontaneous release}] / [\text{cpm maximum release} - \text{cpm spontaneous release}]) \times 100\%$. Spontaneous release was determined by incubating the labeled targets with medium,

20 and maximum release by incubating targets in 1% Triton X-100 solution.

Results

In order to identify latent EBV antigens that are recognized by CD4^+ T cells, $\text{CD8}^+\text{CD2}^+$ peripheral blood mononuclear cells (PBMC) were stimulated for 2 weeks with autologous DCs infected with recombinant vaccinia virus constructs

25 expressing the EBV latent antigens EBNA-1, 3A, 3B, 3C and LMP1, 2. Responses were assessed by the presence of enlarged CD4^+ T cells ("blasts") upon two stimulations with DCs. Specifically, one of a panel of EBV recombinants was used to stimulate the CD4^+ T cells in the first week of culture, and then the cultures were divided in two and restimulated a second week with the original recombinant vaccinia

30 virus or with vvTK⁻ as control. We looked for specific blastogenesis to the primary

-40-

EBV recombinant. All 7 donors showed strong responses to vvEBNA-1 (Table 1; Figure 1A, B). The response to the negative control (vvTK⁻) was weak (Figure 1E) in all but one donor who was excluded from Table 1. All donors responded to influenza infected DCs which were used as a positive control (Figure 1F). A smaller proportion of the 7 donors responded to other vvEBV constructs, *i.e.*, EBNA3B (4/7), EBNA3A (1/7), and LMP1 (3/7) (Table 1A). To ensure that the recombinant vaccinia viruses infected a comparable proportion of the mature DCs, the intracellular expression of the 29 kD vaccinia early protein was measured by flow cytometry. Reproducibly, 40-60% of DCs were infected with the recombinant vaccinia viruses. The reliability of the CD4⁺ recognition of EBNA-1 could be confirmed in an ELISPOT assay for IFN γ secretion, where EBNA-1 was the EBV latency gene that was preferentially recognized (Table 1B). We regard these CD4⁺ T cell responses to EBNA-1 *in vitro* to reflect initial priming by EBV infection of the blood donors *in vivo*, since we did not see blastogenesis in two weeks if we stimulated neonatal T cells from blood cord specimens with EBNA-1. We verified that our donors showed HLA class II diversity since only 2 out of 7 expressed HLA-DR4.

Table 1: CD4⁺ T lymphocyte responses to dendritic cells infected with recombinant vaccinia-EBV vectors.**A: Percentages of blasting CD4⁺ T lymphocytes upon restimulation with specific antigen.**

Donor No.	vvTK ^a	vvEBNA-1	vvEBNA3A	vvEBNA3B	vvEBNA3C	vvLMP1	vvLMP2A	vvBMLF1	Influenza
1	4*	17 (2 ^b)	22 (8)	11 (3)	n.d.	23 (3)	8 (4)	3 (3)	17 (6)
2	2	15 (3)	6 (2)	17 (8)	8 (3)	8 (2)	3 (2)	0 (0)	12 (3)
3	0	11 (6)	0 (0)	19 (8)	0 (0)	30 (18)	0 (0)	0 (0)	28 (10)
4	1	11 (6)	1 (1)	12 (7)	2 (1)	14 (10)	1 (0)	1 (0)	35 (6)
5	1	15 (4)	1 (1)	4 (2)	3 (1)	6 (1)	1 (1)	0 (1)	13 (10)
6	2	8 (3)	n.d.	2 (2)	1 (2)	5 (1)	3 (3)	n.d.	15 (7)
7	3	11 (6)	2 (2)	2 (2)	1 (1)	3 (2)	3 (1)	3 (3)	34 (11)

*Incubations were performed with vaccinia virus infected autologous mature DCs and monitored by flow cytometry after two stimulations.

^aValues in brackets reflect blasted subpopulations that were restimulated with vvTK^a infected DCs as control.**B. IFN γ producing CD4⁺ lymphocytes upon restimulation with specific antigen.**

Donor No.	vvTK ^a	vvEBNA-1	vvEBNA3A	vvEBNA3B	vvEBNA3C	vvLMP1	vvLMP2A	vvBMLF1	Influenza
5	7 \pm 1 ⁵	23 \pm 1 (7 \pm 4 ⁶)	5 \pm 4 (7 \pm 4)	2 \pm 1 (5 \pm 1)	6 \pm 2 (5 \pm 1)	4 \pm 1 (2 \pm 1)	10 \pm 4 (6 \pm 1)	1 \pm 1 (2 \pm 1)	79 \pm 3 (9 \pm 1)
6	3 \pm 1	88 \pm 8 (37 \pm 1)	n.d.	5 \pm 1 (4 \pm 2)	3 \pm 1 (2 \pm 2)	4 \pm 2 (3 \pm 1)	6 \pm 3 (2 \pm 2)	n.d.	88 \pm 3 (27 \pm 6)
7	1 \pm 1	16 \pm 1 (4 \pm 1)	4 \pm 2 (2 \pm 2)	2 \pm 1 (3 \pm 0)	2 \pm 0 (3 \pm 0)	2 \pm 2 (2 \pm 1)	6 \pm 4 (1 \pm 0)	4 \pm 2 (5 \pm 1)	77 \pm 2 (5 \pm 1)

⁵Spot forming cells per 10⁵ cells after overnight incubation with Vaccinia virus infected autologous mature DCs (mean values of triplicates are shown).⁶Values in brackets reflect reactivity against vvTK^a infected DCs as control.

-43-

Additionally, the EBV negative Ramos Burkitt lymphoma cell line, stimulated EBNA-1 specific T cells at a level comparable to DCs infected with vaccinia EBNA-1. However, Ramos cells were not capable of capturing EBNA-1 from allogenic LCL or from high doses of EBNA-1 protein. This data further supports the finding that EBNA-1 is processed endogenously in B cell lines.

In addition to DCs charged with EBNA-1, the CM171198 cell line recognized EBV transformed B-LCL without further addition of antigen. However, the LCL targets had to be matched at the DR4 allele. Thus, the DR4⁺ LCL (LRM and LCL-BM) induced proliferation, but the DR4⁻ cells (LG2 and LCL-DC) did not (Figure 3B). Therefore, EBNA-1 specific CD4⁺ T cells seem to recognize EBV-transformed B cells.

To determine if EBNA-1 also was an antigen for CD4⁺ CTL responses, we stimulated CD8⁺CD2⁺ PBMCs from another (but HLA-DR4⁻) healthy donor, JT, with irradiated autologous B-LCL (which express all known latent EBV antigens (Kieff and Liebowitz, *supra*)) for 14 days with one restimulation after 7 days. In parallel to the stimulation of CD8⁺CD2⁺ T cells, we followed responses to B-LCL in bulk CD2⁺ T cells and in CD4⁺CD2⁺ T cells. ⁵¹Cr-release assays were first performed to document lytic activity against autologous B-LCL, and then the EBV specificity was assessed using DC targets that had been infected with the different recombinant EBV constructs. The content of the stimulated T cell populations was determined by FACS. CD8-depleted responders were enriched for CD4⁺ cells (Figure 4B), CD4-depleted responders were enriched for CD8⁺ cells (Figure 4C) and the bulk T cells had a CD4/CD8 ratio of 1:2 (Figure 4A-C). All contained about 25% CD56⁺ NK cells. All of the stimulated populations, *i.e.*, bulk T cells (Figure 4D) and CD4 (Figure 4E) or CD8 (Figure 4F) enriched cells, killed autologous B-LCL and showed less recognition of the T2 cell line. The latter can be probably attributed to the contaminating NK cells. Remarkably however, the EBV targets for the different T cell responders were quite different (Figure 4, G-I). The CD4⁺ T cells preferentially recognized EBNA-1, EBNA3C and LMP1 (Figure 4H), while CD8⁺ T cells recognized LMP2a (Figure 4I). Bulk T cells recognized EBNA3C, the dominant

-44-

antigen for CD4⁺ cells in this donor, and LMP2a, the dominant antigen for CD8⁺ T cells (Figure 4G).

The CTL function of the stimulated cells was further assessed. As expected, the killing of autologous B-LCL was completely blocked by L243 anti-HLA-DR antibody only when CD4-enriched populations were tested (Figure 5B). LCL-JT killing by CD8-enriched cultures was not blocked by the anti-HLA-DR antibody (Figure 5C), and killing by the bulk T cells only partially inhibited (Figure 5A). The CD4-enriched T cells also lysed EBNA-1 pulsed DCs (Figure 5E), while CD8-enriched cultures did not (Figure 5F) and bulk T cells only weakly (Figure 5D). Therefore, CD4 T cells can lyse autologous B-LCL, and one of the EBV encoded targets is the EBNA-1 latency gene.

To prove that individual CD4⁺ T cells lysed DCs charged with EBNA-1 as well as B-LCL expressing EBNA-1 endogenously, we studied CD4⁺ CTL cloned by limiting dilution from cryopreserved T cells stimulated with autologous B-LCL. The DCs, T cells and autologous B-LCL were derived from leukocyte concentrates. All EBNA-1 specific CD4⁺ CTL could kill the autologous B-LCL (Figure 6A, 6B). Recognition of DCs infected with the vvTK⁻ control vector or LCL721.221, a HLA class I NK target, was poor by these clones (Figure 6A, 6B). Therefore, CD4⁺ T cells can lyse autologous B-LCL, and one target is EBNA-1.

These findings demonstrate CD4⁺ T cell reactivity to EBV latently infected B cells. The nuclear antigen EBNA-1 is repeatedly recognized by CD4⁺ T cells from healthy adults. The CD4⁺ T cells are capable of proliferation, cytokine secretion and cytolytic activity. Other EBV latency antigens that we tested (EBNA3A,3B,3C; LMP1,2; Table 1) can be recognized by CD4⁺ T cells but less reliably than EBNA-1. A single EBNA-1 specific CD4⁺ T cell clone has been described previously (Khanna, *et al.*, Int. Immunol., 9:1537-43. 1997). This clone only killed targets with exogenously processed EBNA-1, probably due to low affinity. The CD4⁺ T cells described here are readily identified in bulk cultures and recognize EBNA-1 processed by exogenous and endogenous routes, even at physiological concentrations as presented on HLA-DR products of transformed B-LCL. However

in more general terms, the successful processing of an endogenous nuclear antigen onto MHC class II is not unusual. When peptides have been eluted from the MHC class II molecules of B cells, only ~20% of the identified peptides are of exogenous origin (Rammensee, *et al.*, in MHC ligands and peptide motifs, Springer: Lands

- 5 Bioscience, Austin, 1997). The endogenously derived peptides also include nuclear antigens like CBF3₃₉₋₇₄ on H2-A^d (Rudensky, *et al.*, Nature, 353:622-7, 1991), Histone H3₁₁₀₋₁₂₇ on HLA-DRB1*0405 (Friede, *et al.*, *supra*, 1996), c-myc₃₇₁₋₃₈₅ on HLA-DRB1*0801 (Chicz, *et al.*, J. Exp. Med., 178:27-47, 1993), and Histone H4₃₁₋₄₅ on HLA-DRB1*1401 (Harris *et al.*, Blood, 87:5104-12, 1996).

- 10 EBNA-1-specific CD4⁺ T cells could provide direct resistance to EBV transformed cells, *e.g.* through their lytic function, or by sustaining the CD8⁺ CTL response to other lymphoma-related EBV products such as LMP-1 and LMP-2. A good deal of circumstantial evidence for CD4⁺ T cell protection against gamma herpesviruses exists in the literature:

- 15 a) The CTL response to EBV in the cotton top tamarin *Sanguinis oedipus* (Cleary *et al.*, Science, 228:72204, 1985) is to a large extent MHC class II restricted (Wilson *et al.*, Clin. Exp. Immunol., 103:199-205, 1996). No MHC class I restricted, EBV-specific CTL have been found to date in this new world monkey, and this species lacks classical MHC class I (although it does express homologues of
- 20 nonclassical class I genes like HLA-G and HLA-F (Watkins, *et al.*, Nature, 346:60-3, 1990)).

b) γ -herpesvirus infection in mice by MHV-68 can be controlled by IFN γ secreting CD4⁺ T cells (Christensen, *et al.*, Proc. Natl. Acad. Sci. USA, 96:5135-5140, 1999).

- 25 c) Control of the growth of Burkitt's lymphoma cells by CD4⁺ T cells has been described in culture (Schattner, *et al.*, Blood, 88:1375-82, 1996), and this lymphoma only expresses a single EBV gene, EBNA-1.

- d) Early in HIV-1 infection, when CD4⁺ T cell counts are still high but CD4⁺ T cell function starts to be compromised, patients can develop Burkitt's
- 30 lymphomas rather than mononucleosis (Levine, Blood, 80:8-20, 1992).

-42-

We then pursued the requisite MHC restriction elements and processing pathways by generating cell lines, initially from an HLA-DR4⁺ donor, CM. We could then test the reactivity of the T cell lines with DCs that had been infected with recombinant vaccinia viruses expressing EBNA-1 or pulsed with soluble EBNA-1 protein. One line, CM171198, was established by stimulating CD8⁺CD2⁺ PBMCs with EBNA-1 processed endogenously. Therefore, the APCs were either the DR4-matched B-LCL LRM or autologous DCs infected with vvEBNA-1ΔGA. The latter construct was deleted of the GA repeat which blocks MHC I presentation and also reduces expression of EBNA-1. The other line, CM110199, was stimulated with exogenously supplied EBNA-1. Therefore, during their final maturation, the DCs were exposed to recombinant EBNA-1 protein expressed either in *E. coli* or in a baculovirus-insect cell-system. After one month of culture, both lines contained predominantly CD4⁺ T cells, 90% in CM171198 (Figure 2A) and 76% in CM110199. Both lines performed similarly in the assays that follow, and therefore, only the data from CM171198 are shown.

The CD4⁺ T cell lines recognized DCs that were infected with vvEBNA-1ΔGA or exogenously supplied recombinant EBNA-1 (Figure 2C, 3A). Reactivity could be measured as IFN γ secretion in ELISPOT assays (Figure 2) or by proliferation assays (Figure 3). The T cell responses were blocked by addition of an anti-HLA-DR antibody, L243, but not by an anti-HLA class I antibody, B-H9 (Figure 2D). As expected, the presence of the GA repeat domain had no effect on this HLA-DR restricted presentation, since DCs infected with full length vvEBNA-1 were also recognized by CM171198, as long as EBNA-1 expression was enhanced by coinfection with vT7 to drive the T7 promotor for EBNA-1 in this construct (Figure 2D). Although there may be gly/ala minus forms of EBNA-1 in DCs infected with full length EBNA-1 expressing vaccinia viruses, we also observe specific T cell recognition of B-LCL (Figure 3), in which only full length EBNA-1 is detected (see, Blake *et al.*, Immunity, 1997, 7:791-802). This further suggests that the gly/ala repeat has no influence on MCH class II processing and presentation.

-46-

e) An impairment of the CD4⁺ response is thought to be responsible for the EBV-induced infectious mononucleosis seen in X-linked lymphoproliferative disease patients who have a mutation or deletion in SAP, an inhibitor of the T cell costimulatory molecule SLAM or CDw150 (Sayos, *et al.*, Nature, 395:462-9, 1998).

f) An antibody response to EBNA-1 is detectable in most donors (Rowe, *et al.*, J. Gen. Virol., 69:1217-28, 1988), consistent with the presence of CD4⁺ helper cells, and EBNA-1 was the only reliable EBV antigen for CD4⁺ responses that we observed.

These observations together with the direct evidence for CD4⁺ T cell responses to EBNA-1, described here, suggest that the latter T cells might provide resistance to Burkitt's, Hodgkin's, and other EBV-associated malignancies in most healthy EBV infected individuals. Our data also suggest that EBNA-1 may be used as an antigen to prevent and treat such malignancies.

For example, immunotherapy of nasopharyngeal carcinoma with EBNA-1 pulsed dendritic cells should result in remission. Immunotherapy appears to be critical for effectively treating nasopharyngeal carcinoma (Tsukuda *et al.*, J. Cancer Res. Clin. Oncol., 120:115, 1993).

The ability to treat nasopharyngeal carcinoma represents a significant step forward in managing this disease. Previous treatments include radiotherapy, surgical resection, and chemotherapy (Sangurineti and Corvo, Oncol. Rep., 6:377, 1999). However, this form of cancer is difficult to treat, and treatment regimens, particularly radiotherapy, cause significant collateral damage to the brain, such as radiation-induced bilateral optic neuropathy (Wijers *et al.*, Stohlenther. Oncol., 175:21, 1999).

**EXAMPLE 2: The EBNA-1-specific CD4⁺ T cells
are primarily T_H1 in function.**

Example 1 discloses a consistent EBNA-1-specific, CD4⁺ T cell response in blood cells from healthy donors. To detect these CD4⁺ T cells, some of which could secrete IFN γ , a 2-week stimulation culture was used in which DCs were the antigen presenting cells and purified CD4⁺ T cells were the responders. This

-47-

example establishes that the EBNA-1-specific CD4⁺ T cells are skewed toward a T_H1 phenotype, and that this response can be detected *in vivo*.

Material and Methods

Dendritic cell and CD4⁺ T cell preparation.

- 5 DCs, see Example 1 above. In some experiments, a rEBNA-1 protein or rPCNA control protein was added to the DC cell culture at the indicated concentrations with the maturation stimulus. For use as target in ⁵¹Cr release assays, DCs were pulsed with either 1 μg/ml of rEBNA-1 protein or of the rPCNA control prior to use as targets. For positive selection of CD4⁺ T cells, CD14⁺ cells were treated with a
10 monoclonal anti-human CD4 antibody conjugated to magnetic microbeads (Miltenyi). T cells and DCs were used fresh or after cryopreservation in FCS and 5% DMSO.

- DC infection with recombinant vaccinia viruses.* Viruses were as previously described (Subklewe, *et al.*, Eur. J. Immunol., 1999, 29:3995-4001). Mature DCs were infected with recombinant vaccinia vectors expressing thymidine
15 kinase (vvTK⁻) as a negative control or vvEBNA-1ΔGA at an MOI of 2 for 1 hour at 37°C, and washed 3 times in medium with 5% human serum. Infection was verified at 6-12 hours by intracellular staining as described previously (Subklewe *et al.*, 1999, *supra*) using VV1-6B6 antibody to a vaccinia early protein followed by FACS analysis. Infection of DCs was uniformly 40-60%.

- 20 *Expression and purification of recombinant EBNA-1 and PCNA control protein.* EBNA-1₄₅₈₋₆₄₁ was inserted in the expression vector pET15b (Novagen, Madison, WI). The vector was transfected into *E. coli* BL21 (DE3) pLysS cells. Proliferating cell nuclear antigen (PCNA) was expressed in *E. coli* BL21 (DE3) pLysS cells (gift from Ming Guo, Cornell University Medical College, New York).
25 Bacterial cultures were grown to an OD₅₉₅ of 0.8 at 37°C. Then EBNA-1 or PCNA expression was induced with 1mM IPTG (GibcoBRL, Grand Island, NY) for 3 hours. After harvesting by centrifugation, the cells were resuspended in 50 mM NaH₂PO₄, 300 mM NaCl and 10 mM imidazole to a volume of 5 ml/g cell pellet. Lysozyme was added to 1 mg/ml for 30 minutes on ice, and the suspension was sonicated for
30 complete lysis. After centrifugation at 20,000xg for 30 minutes at 4°C, the cleared

-48-

supernatant was filtered through a 0.45 μ m filter and 1 ml of Ni-NTA Agarose (Qiagen, Valencia, CA) /10 ml lysate was added. The suspension was rotated for 1 hour at 4°C and packed into a column (Biorad, Hercules, CA). The matrix was washed with 50 mM NaH₂PO₄, 300 mM NaCl and 20 mM imidazole until the flow through OD₂₈₀ was <0.01. The recombinant proteins were then eluted with 50 mM NaH₂PO₄, 300 mM NaCl and 250 mM imidazole. Protein containing fractions were pooled and dialyzed overnight at 4°C against PBS. The protein concentration was determined at OD₂₈₀, purity was determined by SDS PAGE, and identity determined by Western blot with EBNA-1-specific antibody (MAB8173) (CHEMICON Internat. Inc., Temecula, CA) or the 6xH specific antibody AD1.1.10 (R&D Systems).

ELISPOT assay for IFN γ and IL-4 secreting cells. ELISPOT assays were performed as described above. A vvEBNA-1 Δ GA response was considered significant if it were 10 spots greater than the negative control (vvTK⁻) and at least twice that of the negative control.

Expansion of EBNA-1-specific ELISPOT producing, CD4⁺ T cells. Positively-selected CD4⁺ T cells were expanded for 7 days, in medium supplemented with 5% PHS, with vvEBNA-1 Δ GA-infected DCs or vvTK-infected DCs at a DC: T cell ratio of 1:30. In some experiments 5 μ g/ml of W6/32 anti-MHC class I or L243 anti-MHC class II blocking antibody was added on day 0, 3 and 7. At day 7, expanded cells were restimulated with DCs pulsed with 1 μ g/ml rEBNA-1 or control protein, rPCNA, and assayed for IFN γ or IL-4 ELISPOTS. In other experiments, the vaccinia expanded T cells were restimulated with DCs pulsed with the indicated doses of either rEBNA-1 protein or PCNA control protein on day 7.

Generation of EBNA-1-specific cell lines by cytokine secretion in freshly stimulated PBMC. 75 x 10⁶ PBMCs were stimulated with autologous vvEBNA-1 Δ GA-infected DCs at a ratio of 30:1 in medium fortified with 5% PHS for either 7 hours (IFN γ) or 18 hours (IL-4). Then the cells were washed with MACS buffer, centrifuged for 10 minutes at 1800 rpm, and resuspended in cold RPMI containing 10% fetal calf serum (R10) at a concentration of 10⁷ cells/ 80 μ l of media. A primary anti-CD45 antibody, conjugated to either anti-IFN γ or anti-IL-4 (Miltenyi),

-49-

was then added at a ratio of 10 μ l of antibody per 10^7 cells. The cells were then placed on ice for 5 minutes, followed by the addition of warm R10 to a concentration of 5×10^6 cells/ml, and incubated for 45 minutes under continuous rotation at 37°C. Following this incubation, cells were washed with MACS buffer and centrifuged.

5 The pellet was resuspended in 80 μ l of MACS buffer/ 10^7 cells and 10 μ l of secondary antibody to either IFN γ or IL-4 labeled with PE (Milenyi) was added per 10^7 cells. The cells were placed on ice for 10 minutes, and then washed with MACS buffer and centrifugation. A final anti-PE antibody labeled with a paramagnetic microbead (Milenyi) was added at a ratio of 10 μ l/ 10^7 cells for 15 minutes at 4°C. Magnetic separation was performed as above and repeated to increase purity of the recovered cells. The cells were then centrifuged and cultured in medium with 5% PHS and 10^5 irradiated CD14⁺ feeder cells in one well of a 96 well plate. The cells were restimulated weekly; alternating vvEBNA-1 Δ GGA-infected DCs with DCs pulsed with 2 μ g/ml rEBNA-1 at the time of the maturation stimulus (day 5-7). After 3 weeks, 10 U/ml IL-2 (Lymphocult, Biotest, Minneapolis, MN) was added. In this way, we were able to set up lines from cells in fresh blood that secreted IFN γ and IL-4 in response to EBNA-1-pulsed DCs.

FACS and functional analysis of IFN γ and IL-4 cell lines. 3 weeks after initiation of the lines as above, 10^4 cells were stained for 15 minutes on ice with Simultest (CD4 and CD8, BD PharMingen, San Diego, CA) at 1:50 or with PE-labeled CD56 antibody (Becton-Dickenson). After 3 washes and fixation with 4% paraformaldehyde, cells were analyzed on a FACScan (Becton-Dickenson). For cytotoxic activity, the cell lines in triplicate were added to 10^4 targets at the indicated effector: target ratios for 5 hours or 24 hours. The targets were labeled with 50 μ Ci Na₂⁵¹CrO₄ for 1 hour at 37°C, and washed 3 times with R10. To measure cytotoxicity, 50 μ l of culture supernatant was added to 100 μ l of scintillation fluid (Wallac, Finland) in a 96-well sample plate, and radioactivity measured in a γ counter (1450 Microbeta counter, Wallac). Percent specific lysis was calculated by the following formula: $[(\text{cpm experimental well} - \text{cpm spontaneous release}) / (\text{cpm total release} - \text{cpm spontaneous release})] \times 100\%$. Spontaneous release was determined by incubating

-50-

labeled targets in medium alone; total release was determined by incubating targets with 1% Triton X-100.

ELISA for IgG subclasses. 96 well polystyrene plates (Nunc, Rochester, NY) were coated with 1 μ g/well of rEBNA-1 protein, mumps skin antigen USP (Pasteur Merieux Connaught, Swiftwater, PA), tetanus toxoid (Lederle, Philadelphia, PA), candida albicans cell lysate (Allermed Lab, San Diego, CA) in PBS or with PBS alone overnight at 4°C. Plates were blocked with 50 μ l/well 3% non-fat milk powder for 30 minutes followed by 30 minutes in PBS containing 3% BSA. Test plasma samples, diluted 1:10 or 1:100 in 3% BSA, were added for 20 minutes at RT. Plates were washed 3 times with TBST (10 mM Tris, 140mM NaCl, 0.05% Tween 20). Biotin-mouse anti-human IgG1, IgG2, IgG3, IgG4 antibodies (PharMingen) were added at 1:1000 for 20 minutes at RT. Plates were washed 3 times in TBST, avidin-bound biotinylated HRP was added for 20 minutes at RT, followed by TMB substrate (R&D systems) to develop the reaction for 10 minutes at RT and 1M H₂(SO₄) to stop the reaction, and the plates were read in a microplate reader (Dynex, Chantilly, VA).

Results

CD4⁺ T cell responses to EBNA-1 in cultured PBMCs primarily involve IFN γ T_H1 cells. In Example 1, we identified EBNA-1-specific, IFN γ -secreting, CD4⁺ T cells using two, week-long stimulations by DCs infected with recombinant vaccinia EBNA-1 virus (vEBNA-1ΔGA). Here, we assessed if this response could be detected in 1 day and 1 week cultures, and we enumerated both IFN γ and IL-4 secreting cells. At both time points, no EBNA-1-dependent T cells could be detected in cultures stimulated with DCs infected with control vaccinia virus (vTK). With DCs expressing vEBNA-1ΔGA, we found T_H1 cells in 3/9 normal donors after 1 day of culture, but did not find T_H2 cells in any donors (Figures 7A, B). In 1 week cultures, 8/9 donors demonstrated an expansion of IFN γ cells, but only 1/9 had EBNA-1 dependent IL-4 secretors (Figures 7A, B). In the one IL-4 secretor, the number of IFN γ ELISPOTS was three times greater than the IL-4 ELISPOTS.

-51-

Therefore, in most donors, EBNA-1 responsive CD4⁺ T cells have a T_H1 phenotype, secreting IFN γ and not IL-4. This is a surprising and unexpected result considering that others have found the EBNA-1-specific T cell response is T_H2 in phenotype, rather than T_H1 (see, Steigerwald-Mullen, *et al.*, J. Virol., 2000, 74:6748-6759).

To verify that these CD4⁺ T cell responses were MHC class II restricted, we stimulated T cells with vvEBNA-1 Δ GA-infected DCs for one week in the absence or presence of blocking antibodies to MHC Class I (W6/32) or HLA-DR (L243). When IFN γ -secreting cells were enumerated by ELISPOT, only the L243 monoclonal antibody decreased responses, by a range of 88-100% in 3 experiments. We conclude that CD4⁺ T cell responses to EBNA-1 are primarily MHC II restricted and of the T_H1 type.

The experiments in Figure 7 used vaccinia vectors both to expand the EBNA-1-specific cells for one week and for restimulation in the ELISPOT assay for one day. Therefore, we then tested the efficacy of purified EBNA-1 protein as antigen in the ELISPOT assay. A recombinant EBNA-1 protein consisting of the amino acids 458-641 of the EBNA-1 sequence or rPCNA, as a control protein, were extracted from transformed *E. coli* cultures after IPTG induction. The extract was dialyzed overnight against PBS and was checked for purity and specificity by SDS PAGE and Western blot (Figure 8A). These proteins were pulsed onto DCs in varying concentrations and used to read-out IFN γ ELISPOTs after a one week expansion using vvEBNA-1 Δ GA-infected DCs. Figure 8B shows the dose response curve seen with the titration of rEBNA-1 protein as compared to the PCNA control. The inset graph compares the response of vvEBNA-1 Δ GA expanded cells restimulated with vvTK⁻ or vvEBNA-1 Δ GA. A dosage of only 1 μ g/ml of rEBNA-1 protein pulsed onto DCs gives a response that is comparable to that of the recombinant vaccinia EBNA-1. This result demonstrates that EBNA-1-specific T_H1 cells are capable of responding to very low doses of EBNA-1.

EBNA-1-specific, cytokine-secreting cells isolated directly ex vivo are primarily CD4⁺. Since the EBNA-1-specific ELISPOT responses from most donors

-52-

required a week's culture of CD4⁺ T cells with DCs, which make high levels of the TH1 skewing cytokine IL-12 (Cella, *et al.*, J. Exp. Med., 1996, 184:747-752; Koch, *et al.*, J. Exp. Med., 1996, 184:741-746), we isolated EBNA-1-specific cells directly *ex vivo*. Fresh PBMCs from 6 donors were stimulated for 7 hours (IFN γ) or 18 hours (IL-4) with vvEBNA-1 Δ GA-infected DCs. Then the cells were labeled with anti-CD45 antibody conjugated to either anti-IFN γ or IL-4 antibody. In this way, cells that were secreting cytokine would capture a second antibody specific to another epitope of IFN γ or IL-4, labeled with PE. A final anti-PE antibody conjugated to a magnetic microbead was added and the cells selected in a magnetic field. The recovered cells were expanded by weekly restimulations, alternating vvEBNA-1 Δ GA-infected DCs with DCs pulsed with rEBNA-1 protein.

EBNA-1-specific, IFN γ secreting cell lines were established in all 6 donors, and IL-4 secreting lines in 3/6 donors. When the lines were analyzed by FACS (for CD56, CD4 or CD8), both IFN γ and IL-4 lines consisted primarily of CD4⁺ cells. Figure 9 shows IFN γ secreting lines from 3 donors, i.e., >90% of the cells expressed CD4 and <2% CD8 or CD56 (data not shown). Likewise three EBNA-1-specific, IL-4 secreting lines consisted of >90% CD4 and <2% CD8 or CD56 cells (data not shown). We conclude that EBNA-1-specific, cytokine-secreting CD4⁺ T cells in healthy adults are already differentiated *in vivo* (Figure 9), but these cells typically must be expanded for 1 week with autologous DCs *in vitro* to be detected in ELISPOT assays (Figure 7).

EBNA-1-specific T_H1 and not T_H2 CD4 T cells are able to lyse

EBNA-1-expressing DCs. We have shown that EBNA-1-specific T cell lines can kill autologous B-LCL and DCs pulsed with different sources of EBNA-1 protein. We assessed if this function was primarily expressed by T_H1 lines, as has been observed with CD4⁺ T cells specific for other antigens (Erb, *et al.*, Cell Immunol., 1991, 135:232-44; Erb, *et al.*, J. Immunol., 1990, 144:790-795; Del Prete, *et al.*, J. Exp. Med., 1991, 174:809-13; Nishimura, *et al.*, J. Exp. Med., 1999, 190:617-628). IFN γ cell lines from 5 donors and IL-4 lines from 3 donors were studied simultaneously in

-53-

cytotoxicity and ELISPOT assays. The cell lines, which had been isolated from fresh PBMCs on the basis of EBNA-1 dependent, IFN γ or IL-4 production, maintained a T_H1 or T_H2 polarity after weeks of stimulation in the absence of exogenous IL-12 or IL-4 (Figure 10A, B). When we tested lysis of vvEBNA-1AGA-infected DCs, DCs loaded with rEBNA-1 protein or autologous B-LCL by ⁵¹Cr release assay, only T_H1 lines lysed EBNA-1-expressing targets over the controls, though the T_H2 cell lines recognized EBNA-1-expressing DCs with the secretion of IL-4. Figure 10C, D shows this data at graded effector:target ratios, while Figure 10E summarizes killing at an E:T ratio of 10:1 for several lines. T_H1 but not T_H2 cell lines had cytolytic activity (Figure 10E).

EBNA-1-specific IgG subclasses in vivo reflect T_H1 immunity. To assess the relative activity of EBNA-1 specific T_H1 and T_H2 cells in vivo, we monitored the IgG subclass of the antibody response. T_H1 cytokines skew antibody responses towards the IgG1 isotype, and T_H2 toward IgG4, although the IgG subclass distribution in humans may not be as strictly biased as described for mice (Bonifacio, *et al.*, J. Immunol., 1999, 163:525-32; Sousa, *et al.*, Clin. Exp. Immunol., 1998, 111:48-55; Hussain, *et al.*, Immunology., 1999, 98:238-43). An ELISA assay for IgG subclasses was used to describe the isotype of EBNA-1-specific IgG antibodies in 7 donors. As antigen, we used either a commercially-available gly-ala repeat sequence from EBNA-1, or the C-terminal rEBNA-1 protein expressed by *E. coli* vectors. All 7 donors showed a clear predominance of IgG1 antibodies in their EBNA-1 response (Figure 11). This contrasts to responses to the three other antigens that we tested (mumps skin antigen, tetanus toxoid and candida lysate; Figure 11), although two donors did make a strong IgG1 response to tetanus toxoid. The antibody data, coupled with the ready detection of IFN γ secreting T cells in fresh and 1 week cultures of PBMC, indicates that the response to EBNA-1 *in vivo* in healthy EBV carriers is consistently T_H1 in type.

Discussion

The new data indicate that healthy EBV carriers consistently make a T_H1 type response to EBNA-1 *in vivo*. These T cells are present in the blood and

-54-

likely account for the unambiguous skewing of EBNA-1 antibody responses to the IgG1 isotype (Figure 10). Since B cells and B cell lines are not known to actively produce IL-12, or to bias the CD4⁺ T cell response towards T_H1, we suspect that DCs are responsible for skewing T cells in this fashion. It is now known that human DCs efficiently process EBNA-1 from dying EBV-infected B cells (Münz, *et al.*, J. Exp. Med., 2000, 191:1649-60), and that mouse DCs skew T cells towards the T_H1 type in vivo (Pulendran, *et al.*, Proc. Natl. Acad. Sci. USA, 1999, 96:1036-1041; Maldonado- Lopez, *et al.*, J. Exp. Med., 1999, 189:587-592). If human B cells have to undergo a lytic infection to enable DCs to present EBNA-1 *in vivo*, one would expect a delay in the development of EBNA-1-specific antibodies, as is typical of EBV infection (Rickinson, and Kieff, Epstein-Barr Virus. In Virology. B.N. Fields, D.M. Knipe, and P.M. Howley, editors. 1996, Lippincott-Raven, Philadelphia. 2397-2446). Therefore, we suggest that the T_H1 response to EBNA-1 reflects a dominant role for DCs relative to infected B cells, as the direct inducers of EBNA-1 immunity.

CD4⁺ T cells are important in resistance to virus infections and tumors. In HIV-1 infection, strong CD4⁺ T cell responses are found in long term nonprogressors (Rosenberg, *et al.*, Science, 1997, 278:1447-1450). This minority of HIV-infected patients have high CD4⁺ T cell counts and low viral loads without anti-retroviral therapy. They also display more vigorous CD4⁺ T cell proliferative responses to HIV p24 and gp160 protein. The role of CD4⁺ T cells in chronic viral infection has been more directly assessed in mice (Cardin, *et al.*, J. Exp. Med., 1996, 184:863-871). Primary infection with the murine MHV-68 γ -herpesvirus resolves similarly in MHC class II^{-/-} and ^{+/+} mice. However, 3 weeks after the initial infection, the virus recrudesces in MHC II knock-out mice which then develop wasting and within 4 months, the majority of the mice die. This occurs in spite of the fact that initial viral clearance takes place, apparently through CD8⁺ T cell cytotoxicity. Analogous findings have been made in studies of CD8⁺ effector function towards LCMV infection in CD4^{-/-} and wild type mice (Zajac, *et al.*, J. Exp. Med., 1998, 188:2205-2213). The CD4^{-/-} mice fail to eliminate an LCMV variant that causes more

widespread and chronic infection, and the variant-specific CD8⁺ T lymphocytes are non-functional (as measured by IFN γ secretion), despite expression of lymphocyte activation markers. Together these results are part of an emerging consensus that CD4⁺ T cells maintain effective CD8⁺ T cell function against viruses (reviewed in Kalams and Walker, *J. Exp. Med.*, 1998, 188:2199-2204) and tumors (reviewed in Toes, *et al.*, *J. Exp. Med.*, 1999, 189:753-756).

The mechanism underlying this role for CD4⁺ T cells likely entails improved function of antigen presenting cells, especially DCs (Schoenberger, *et al.*, *Nature*, 1998, 393:480-483; Ridge, *et al.*, *Nature*, 1998, 393:474-478; Bennett, *et al.*, *Nature*, 1998, 393:478-480). The DCs, following interaction with CD4⁺ T cells, become more effective stimulators of CD8⁺ T cells, both in their initial expansion and maintenance. CD40L, which is expressed more abundantly on activated CD4⁺ than CD8⁺ T cells, is strongly implicated as the stimulus for DCs. CD40 is abundant on DCs, and its ligation mediates several critical steps in DC development and function. This includes their generation from CD34⁺ progenitors (Flores-Romo, *et al.*, *J. Exp. Med.*, 1997, 185:341-349), mobilization from peripheral tissues (Moodycliffe, *et al.*, *J. Exp. Med.*, 2000, 191:2011-20), maturation (Caux, *et al.*, *J. Exp. Med.*, 1994, 180:1263-1272), survival (Josien, *et al.*, *J. Exp. Med.*, 2000, 191:495-501), and cytokine secretion, particularly IL-12 (Cella, *et al.*, *J. Exp. Med.*, 1996, 184:747-752; Koch, *et al.*, *J. Exp. Med.*, 1996, 184:741-746).

T_H1 are more important than T_H2 CD4⁺ cells in resistance to viruses and tumors. In humans, it has been proposed that T_H1 CD4⁺ T lymphocytes resist primary CMV infection in renal transplant recipients (Rentenaar, *et al.*, *J. Clin. Invest.*, 2000, 105:541-8). CMV seronegative recipients of CMV-positive kidneys were monitored for CMV-specific immune responses after transplantation. In all patients evaluated, a polarized T_H1 response was observed. These patients recovered from acute infection without signs of chronic CMV disease, despite immunosuppressive therapy. Furthermore, studies of mouse models emphasize the importance of T_H1 CD4⁺ cells in immunological resistance. When ovalbumin was expressed in tumors as a surrogate antigen, adoptive transfer of ovalbumin-specific

T_H1 cells led to stronger $CD8^+$ T cell memory than adoptive transfer of T_H2 cells with the identical T cell receptor for the antigen (Nishimura, *et al.*, 1999, *supra*). Neonatal mice immunized with an influenza subunit vaccine in combination with IL-12 exhibited enhanced T_H1 cytokine expression and demonstrated 100% survival after challenge with influenza virus in comparison to a 55% survival rate among neonatal mice immunized with the influenza subunit vaccine alone (Arulanandam, *et al.*, J. Immunol., 2000, 164:3698-704). A recent study employed adoptive transfer into $TCR\beta^{-/-}\delta^{-/-}$ mice of T_H1 and T_H2 cells expressing an identical TCR transgene specific for the vesicular stomatitis virus (VSV) glycoprotein (Maloy, *et al.*, J. Exp. Med., 2000, 191:2159-70). Both T_H1 and T_H2 T cells conferred systemic protection against VSV infection, most likely through the elaboration of neutralizing antibodies. However, only the T_H1 $CD4^+$ T cells were able to protect against lethal intranasal infection and provoke a DTH response. These examples delineate a vital role for T_H1 $CD4^+$ T lymphocytes in the control of primary viral infections and the development and maintenance of immunological memory.

There are several possible mechanisms for the protective function of T_H1 $CD4^+$ T cells. $IFN\gamma$ secretion has effects on a variety of cell types, including B cells, in which it influences switching of the IgG subclasses to IgG2a in mice (Arulanandam, *et al.*, 2000, *supra*) and to IgG1 in humans (Bonifacio, *et al.*, 1999, *supra*; Sousa, *et al.*, 1998, *supra*; Hussain, *et al.*, 1999, *supra*). These antibodies can enhance the efficiency of opsonization and complement fixation. Serum from neonatal mice given IL-12 prior to vaccination against influenza demonstrated heightened levels of influenza-specific, T_H1 -dependent, IgG2a antibodies (Arulanandam, *et al.*, 2000, *supra*). Passive transfer of serum from these mice to B cell deficient mice conferred better protection to live influenza challenge than serum from mice vaccinated without IL-12, which had mainly IgG1 influenza-specific antibodies. In other studies, however, the quantity of IgG antibody and not the subclass was important in conferring protection to viral challenge (Bachmann, *et al.*, Science, 1997, 276:2024-2027). $TCR\beta^{-/-}\delta^{-/-}$ mice infused with either T_H1 or T_H2 transgenic VSV-specific $CD4^+$ T cells elaborated polarized IgG antibodies to VSV,

-57-

both of which conferred protective immunity (Maloy, *et al.*, 2000, *supra*). Interestingly, we find that the human IgG1, T_H1-type antibody response to EBNA-1 is very large relative to other antibody responses (Figure 11), consistent with the capacity of DCs to orchestrate strong antibody production from B cells (Fayette, *et al.*, J. Exp. Med., 1997, 185:1909-1918; Dubois, *et al.*, J. Exp. Med., 1997, 185:941-951).

T_H1 CD4⁺ T cells could also control viral infections through cytotoxicity. In the current study, only T_H1 cells could lyse EBNA-1-expressing targets (Figure 10). The restriction of cytotoxicity to T_H1 cells has been described previously (Bonifacio, *et al.*, 1999, *supra*; Sousa, *et al.*, 1998, *supra*; Hussain, *et al.*, 1999, *supra*). Likewise, in a model in which ovalbumin-specific T_H1 and T_H2 cells were generated from TCR transgenic mice and tested for their ability to lyse murine tumor cells expressing ovalbumin, only the IFN γ -secreting CD4⁺ T cells lysed tumor cells (Hussain, *et al.*, 1999, *supra*).

Importantly, T_H1 and T_H2 cells differ in their ability to home to sites of infection (Austrop, *et al.*, Nature, 1997, 385:81-83; O'Garra, *et al.*, Curr. Biol., 1998, 8:R646-9). T_H1 cells but not T_H2 cells migrate in response to the corresponding chemokines (MCP-1, Mig and IP-10) that are produced in infected tissues (Maloy, *et al.*, 2000, *supra*). This is due to differential expression of chemokine receptors, T_H1 cells expressing CCR2, CCR5 and CXCR3 and T_H2 cells, CCR4.

As mentioned above, in a murine model of tumor immunity, adoptive transfer of either T_H1 or T_H2 cells eradicated tumors, but only T_H1 cells generated immunological memory to tumor rechallenge (Hussain, *et al.*, 1999, *supra*). Interestingly, the mechanisms of tumor eradication appeared very different between the two types of helper cells. In mice receiving T_H1 cells, the tumor was infiltrated mainly by lymphocytes. Conversely, the mice that received T_H2 cells demonstrated a tumor infiltrate marked by eosinophils and neutrophils.

Because the EBNA-1 protein is the sole EBV latency protein expressed in all forms of EBV-associated cancers and it elicits a T_H1 CD4⁺ T cell response, this protein provides a new focus for vaccination, especially in children, and for

-58-

immunotherapy for EBV-associated malignancies. Additionally, therapy targeted at polarizing EBNA-1-specific CD4⁺ T lymphocytes towards T_H1 response would best involve targeting of EBNA-1 to immunogenic DCs.

5

* * *

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying Figures. Such modifications are intended to fall within the scope of the appended claims.

10

It is further to be understood that all values are approximate, and are provided for description.

All patents, patent applications, publications, and other materials cited herein are hereby incorporated herein reference in their entireties.

15

WHAT IS CLAIMED IS:

- 1 1. A vaccine comprising an immunogenic EBNA-1 polypeptide
2 and an adjuvant acceptable for use in a human.
- 1 2. The vaccine of claim 1, wherein the immunogenic polypeptide
2 is expressed in *E. coli*.
- 1 3. The vaccine of claim 1, wherein the immunogenic polypeptide
2 is expressed in insect cells using a baculovirus vector.
- 1 4. The vaccine of claim 1, wherein the immunogenic polypeptide
2 is a fusion polypeptide comprising an amino acid sequence of EBNA-1 and a
3 heterologous amino acid sequence.
- 1 5. An expression vector for expression in humans comprising a
2 sequence encoding an immunogenic EBNA-1 polypeptide, operably associated with
3 an expression control sequence.
- 1 6. The vector of claim 6 which preferentially targets dendritic
2 cells.
- 1 7. The vector of claim 6, wherein the polypeptide is a fusion
2 polypeptide comprising an amino acid sequence of EBNA-1 and a heterologous
3 amino acid sequence.
- 1 8. The vector of claim 6 which is a viral vector.
- 1 9. The vector of claim 8, wherein the viral vector is a genetically
2 engineered vaccinia virus.

1 10. A method for protecting a subject from infection by Epstein
2 Barr Virus, which method comprises delivering an immunologically effective amount
3 of an immunogenic EBNA-1 polypeptide to the subject.

1 11. The method according to claim 10, further comprising
2 delivering an immunostimulatory amount of an immune activating or inflammatory
3 cytokine to the subject.

1 12. A method for protecting a subject from infection by Epstein
2 Barr Virus comprising delivering an immuno-protective amount of the expression
3 vector of claim 5 to the subject.

1 13. The method according to claim 12, further comprising
2 delivering an immunostimulatory amount of an immune activating or inflammatory
3 cytokine to the subject.

1 14. The method according to claim 12, wherein delivering the
2 expression vector comprises transplanting dendritic cells harboring the expression
3 vector into the subject.

1 15. The method according to claim 12, wherein the expression
2 vector targets dendritic cells *in vivo*.

1 16. The method according to claim 10, to prevent or treat an EBV-
2 associated neoplasm.

1 17. The method according to claim 16, wherein the neoplasm is
2 nasopharyngeal carcinoma.

-61-

- 1 18. The method according to claim 12, to prevent or treat an EBV-
2 associated neoplasm.
- 1 19. The method according to claim 18, wherein the neoplasm is
2 nasopharyngeal carcinoma.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000
1001
1002
1003
1004
1005
1006
1007
1008
1009
1010
1011
1012
1013
1014
1015
1016
1017
1018
1019
1020
1021
1022
1023
1024
1025
1026
1027
1028
1029
1030
1031
1032
1033
1034
1035
1036
1037
1038
1039
1040
1041
1042
1043
1044
1045
1046
1047
1048
1049
1050
1051
1052
1053
1054
1055
1056
1057
1058
1059
1060
1061
1062
1063
1064
1065
1066
1067
1068
1069
1070
1071
1072
1073
1074
1075
1076
1077
1078
1079
1080
1081
1082
1083
1084
1085
1086
1087
1088
1089
1090
1091
1092
1093
1094
1095
1096
1097
1098
1099
1100
1101
1102
1103
1104
1105
1106
1107
1108
1109
1110
1111
1112
1113
1114
1115
1116
1117
1118
1119
1120
1121
1122
1123
1124
1125
1126
1127
1128
1129
1130
1131
1132
1133
1134
1135
1136
1137
1138
1139
1140
1141
1142
1143
1144
1145
1146
1147
1148
1149
1150
1151
1152
1153
1154
1155
1156
1157
1158
1159
1160
1161
1162
1163
1164
1165
1166
1167
1168
1169
1170
1171
1172
1173
1174
1175
1176
1177
1178
1179
1180
1181
1182
1183
1184
1185
1186
1187
1188
1189
1190
1191
1192
1193
1194
1195
1196
1197
1198
1199
1200
1201
1202
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
1213
1214
1215
1216
1217
1218
1219
1220
1221
1222
1223
1224
1225
1226
1227
1228
1229
1230
1231
1232
1233
1234
1235
1236
1237
1238
1239
1240
1241
1242
1243
1244
1245
1246
1247
1248
1249
1250
1251
1252
1253
1254
1255
1256
1257
1258
1259
1260
1261
1262
1263
1264
1265
1266
1267
1268
1269
1270
1271
1272
1273
1274
1275
1276
1277
1278
1279
1280
1281
1282
1283
1284
1285
1286
1287
1288
1289
1290
1291
1292
1293
1294
1295
1296
1297
1298
1299
1300
1301
1302
1303
1304
1305
1306
1307
1308
1309
1310
1311
1312
1313
1314
1315
1316
1317
1318
1319
1320
1321
1322
1323
1324
1325
1326
1327
1328
1329
1330
1331
1332
1333
1334
1335
1336
1337
1338
1339
1340
1341
1342
1343
1344
1345
1346
1347
1348
1349
1350
1351
1352
1353
1354
1355
1356
1357
1358
1359
1360
1361
1362
1363
1364
1365
1366
1367
1368
1369
1370
1371
1372
1373
1374
1375
1376
1377
1378
1379
1380
1381
1382
1383
1384
1385
1386
1387
1388
1389
1390
1391
1392
1393
1394
1395
1396
1397
1398
1399
1400
1401
1402
1403
1404
1405
1406
1407
1408
1409
1410
1411
1412
1413
1414
1415
1416
1417
1418
1419
1420
1421
1422
1423
1424
1425
1426
1427
1428
1429
1430
1431
1432
1433
1434
1435
1436
1437
1438
1439
1440
1441
1442
1443
1444
1445
1446
1447
1448
1449
1450
1451
1452
1453
1454
1455
1456
1457
1458
1459
1460
1461
1462
1463
1464
1465
1466
1467
1468
1469
1470
1471
1472
1473
1474
1475
1476
1477
1478
1479
1480
1481
1482
1483
1484
1485
1486
1487
1488
1489
1490
1491
1492
1493
1494
1495
1496
1497
1498
1499
1500
1501
1502
1503
1504
1505
1506
1507
1508
1509
1510
1511
1512
1513
1514
1515
1516
1517
1518
1519
1520
1521
1522
1523
1524
1525
1526
1527
1528
1529
1530
1531
1532
1533
1534
1535
1536
1537
1538
1539
1540
1541
1542
1543
1544
1545
1546
1547
1548
1549
1550
1551
1552
1553
1554
1555
1556
1557
1558
1559
1560
1561
1562
1563
1564
1565
1566
1567
1568
1569
1570
1571
1572
1573
1574
1575
1576
1577
1578
1579
1580
1581
1582
1583
1584
1585
1586
1587
1588
1589
1590
1591
1592
1593
1594
1595
1596
1597
1598
1599
1600
1601
1602
1603
1604
1605
1606
1607
1608
1609
1610
1611
1612
1613
1614
1615
1616
1617
1618
1619
1620
1621
1622
1623
1624
1625
1626
1627
1628
1629
1630
1631
1632
1633
1634
1635
1636
1637
1638
1639
1640
1641
1642
1643
1644
1645
1646
1647
1648
1649
1650
1651
1652
1653
1654
1655
1656
1657
1658
1659
1660
1661
1662
1663
1664
1665
1666
1667
1668
1669
1670
1671
1672
1673
1674
1675
1676
1677
1678
1679
1680
1681
1682
1683
1684
1685
1686
1687
1688
1689
1690
1691
1692
1693
1694
1695
1696
1697
1698
1699
1700
1701
1702
1703
1704
1705
1706
1707
1708
1709
1710
1711
1712
1713
1714
1715
1716
1717
1718
1719
1720
1721
1722
1723
1724
1725
1726
1727
1728
1729
1730
1731
1732
1733
1734
1735
1736
1737
1738
1739
1740
1741
1742
1743
1744
1745
1746
1747
1748
1749
1750
1751
1752
1753
1754
1755
1756
1757
1758
1759
1760
1761
1762
1763
1764
1765
1766
1767
1768
1769
1770
1771
1772
1773
1774
1775
1776
1777
1778
1779
1780
1781
1782
1783
1784
1785
1786
1787
1788
1789
1790
1791
1792
1793
1794
1795
1796
1797
1798
1799
1800
1801
1802
1803
1804
1805
1806
1807
1808
1809
1810
1811
1812
1813
1814
1815
1816
1817
1818
1819
1820
1821
1822
1823
1824
1825
1826
1827
1828
1829
1830
1831
1832
1833
1834
1835
1836
1837
1838
1839
1840
1841
1842
1843
1844
1845
1846
1847
1848
1849
1850
1851
1852
1853
1854
1855
1856
1857
1858
1859
1860
1861
1862
1863
1864
1865
1866
1867
1868
1869
1870
1871
1872
1873
1874
1875
1876
1877
1878
1879
1880
1881
1882
1883
1884
1885
1886
1887
1888
1889
1890
1891
1892
1893
1894
1895
1896
1897
1898
1899
1900
1901
1902
1903
1904
1905
1906
1907
1908
1909
1910
1911
1912
1913
1914
1915
1916
1917
1918
1919
1920
1921
1922
1923
1924
1925
1926
1927
1928
1929
1930
1931
1932
1933
1934
1935
1936
1937
1938
1939
1940
1941
1942
1943
1944
1945
1946
1947
1948
1949
1950
1951
1952
1953
1954
1955
1956
1957
1958
1959
1960
1961
1962
1963
1964
1965
1966
1967
1968
1969
1970
1971
1972
1973
1974
1975
1976
1977
1978
1979
1980
1981
1982
1983
1984
1985
1986
1987
1988
1989
1990
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
2022
2023
2024
2025
2026
2027
2028
2029
2030
2031
2032
2033
2034
2035
2036
2037
2038
2039
2040
2041
2042
2043
2044
2045
2046
2047
2048
2049
2050
2051
2052
2053
2054
2055
2056
2057
2058
2059
2060
2061
2062
2063
2064
2065
2066
2067
2068
2069
2070
2071
2072
2073
2074
2075
2076
2077
2078
2079
2080
2081
2082
2083
2084
2085
2086
2087
2088
2089
2090
2091
2092
2093
2094
2095
2096
2097
2098
2099
2100
2101
2102
2103
2104
2105
2106
2107
2108
2109
2110
2111
2112
2113
2114
2115
2116
2117
2118
2119
2120
2121
2122
2123
2124
2125
2126
2127
2128
2129
2130
2131
2132
2133
2134
2135
2136
2137
2138
2139
2140
2141
2142
2143
2144
2145
2146
2147
2148
2149
2150
2151
2152
2153
2154
2155
2156
2157
2158
2159
2160
2161
2162
2163
2164
2165
2166
2167
2168
2169
2170
2171
2172
2173
2174
2175
2176
2177
2178
2179
2180
2181
2182
2183
2184
2185
2186
2187
2188
2189
2190
2191
2192
2193
2194
2195
2196
2197
2198
2199
2200
2201
2202
2203
2204
2205
2206

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
22 February 2001 (22.02.2001)

PCT

(10) International Publication Number
WO 01/12215 A3(51) International Patent Classification: C12N 15/38,
C07K 14/05, A61K 39/245, 48/00

(21) International Application Number: PCT/US00/22106

(22) International Filing Date: 10 August 2000 (10.08.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data: 60/148,971 13 August 1999 (13.08.1999) US

(71) Applicant (for all designated States except US): THE
ROCKEFELLER UNIVERSITY [US/US]; 1230 York
Avenue, New York, NY 10021-6399 (US).

(72) Inventor; and

(75) Inventor/Applicant (for US only): STEINMAN, Ralph,
M. [US/US]; 62 North Avenue, Westport, CT 06880 (US).(74) Agents: FEHLNER, Paul, F. et al.; Darby & Darby P.C.,
805 Third Avenue, New York, NY 10022-7513 (US).(81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,
DE, DK, DM, EE, ES, FI, GB, GD, GE, HR, HU, ID, IL,
IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU,
LV, MA, MD, MG, MK, MN, MW, MX, MY, NZ, NO, NZ, PL,
PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ,
UA, UG, US, UZ, VN, YU, ZA, ZW.(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

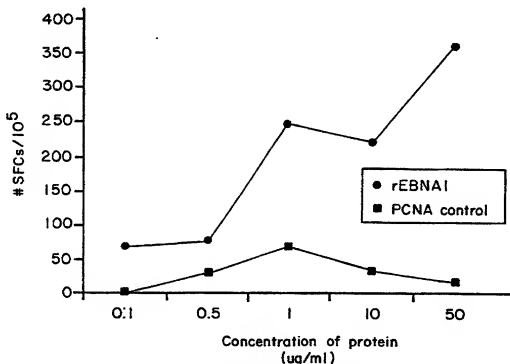
Published:

— with international search report

(88) Date of publication of the international search report:
23 August 2001

[Continued on next page]

(54) Title: PROTECTIVE ANTIGEN OF EPSTEIN BARR VIRUS



(57) Abstract: The present invention relates to the identification of a subunit vaccine to prevent or treat infection of Epstein Barr Virus. In particular, EBNA-1 was identified as a vaccine antigen. In a specific embodiment, a purified protein corresponding to EBNA-1 elicited a strong CD4⁺ T cell response. The responsive CD4⁺ T cell are primarily T_H1 in function. EBNA-1 is an attractive candidate for a protective vaccine against EBV, and for immunotherapy of EBV infection and neoplasms, particularly with dendritic cells charged with EBNA-1.

WO 01/12215 A3

1/24

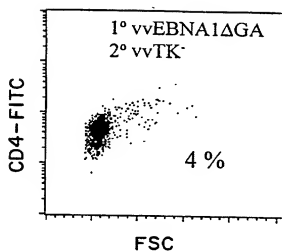


FIG. 1A

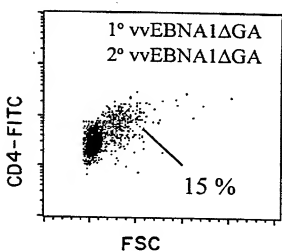


FIG. 1B

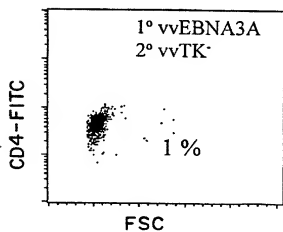


FIG. 1C

2/24

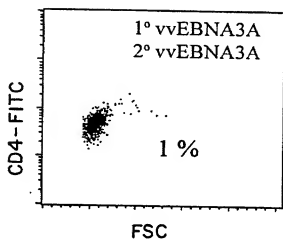


FIG. ID

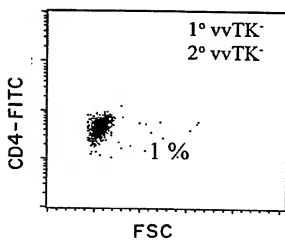


FIG. IE

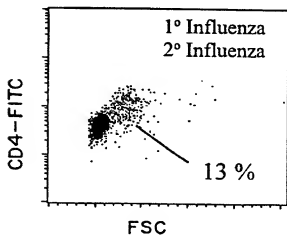


FIG. IF

3/24

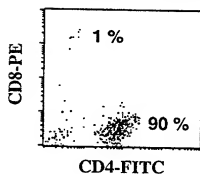
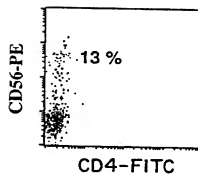
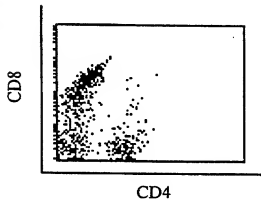
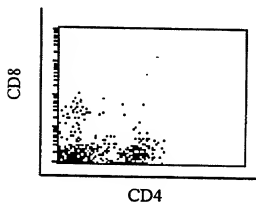
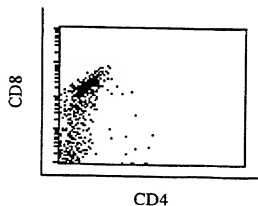
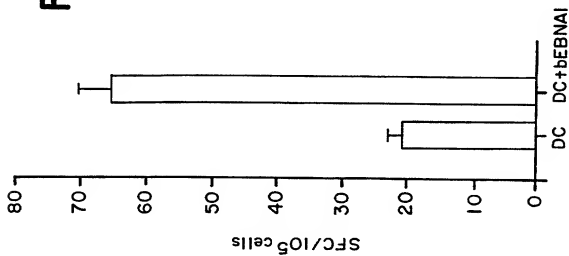
FIG. 2A**FIG. 2B****FIG. 4A**CD2⁺PBMC**FIG. 4B**CD8⁻CD2⁺PBMC**FIG. 4C**CD4⁺CD2⁺PBMC

FIG. 2D



5/24

FIG. 3B

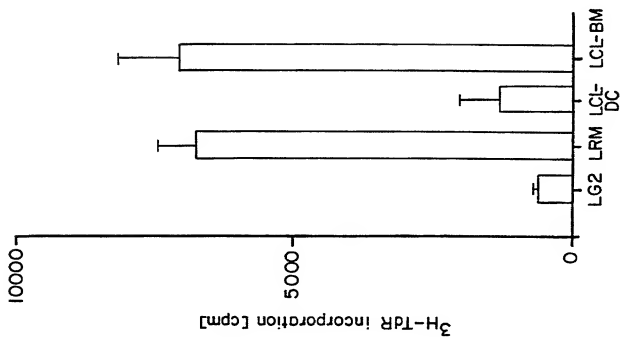
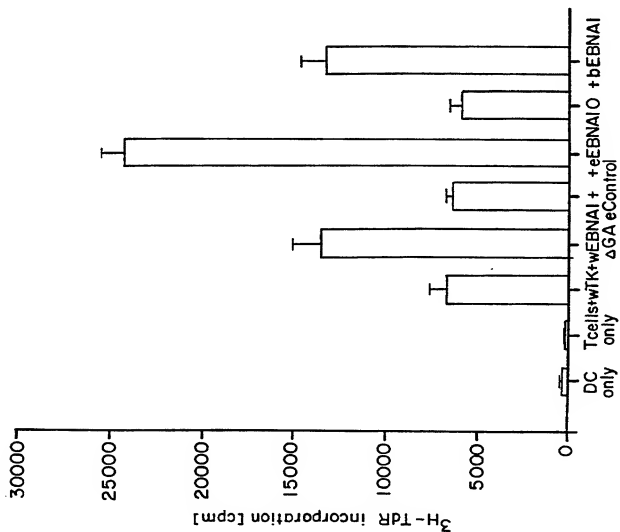


FIG. 3A



6/24

FIG. 4D

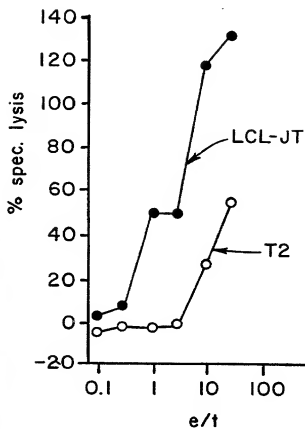


FIG. 4E

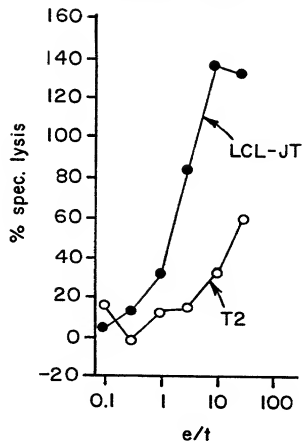
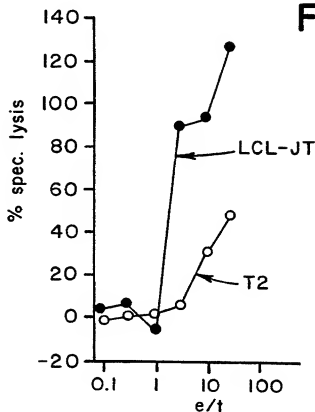
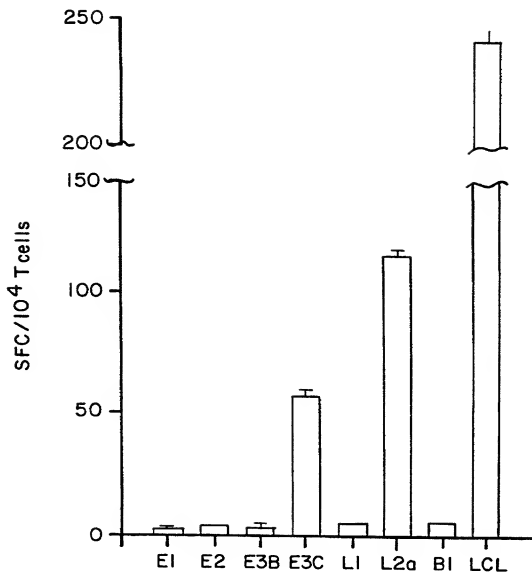


FIG. 4F



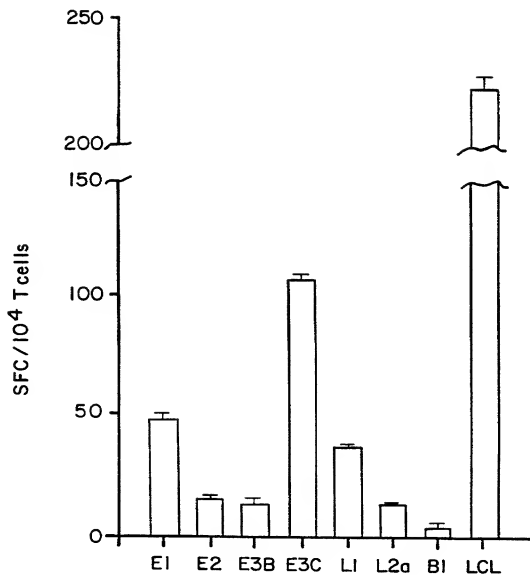
7/24

FIG. 4G



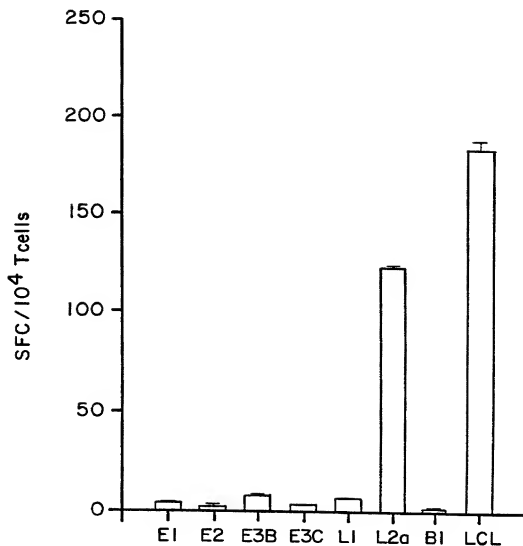
8/24

FIG. 4H



9/24

FIG. 4I



10/24

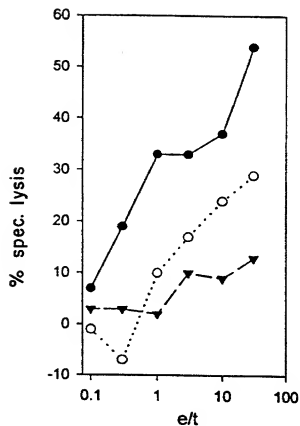


FIG. 5A

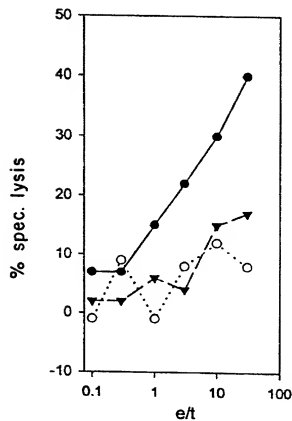
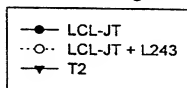


FIG. 5B

LCL targets:



11/24

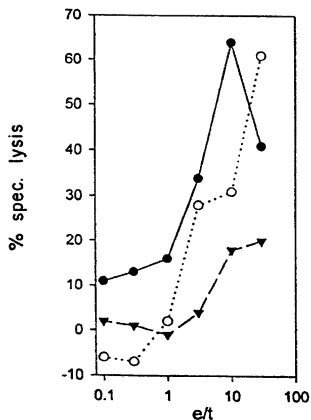


FIG. 5C

LCL targets:

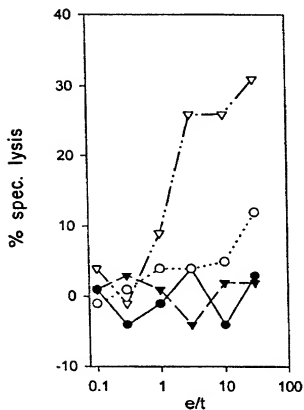
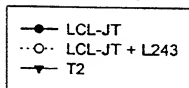
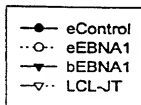


FIG. 5D

DC targets:



12/24

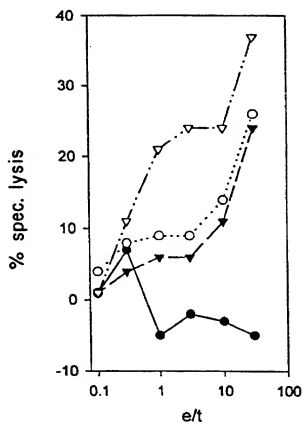


FIG. 5E

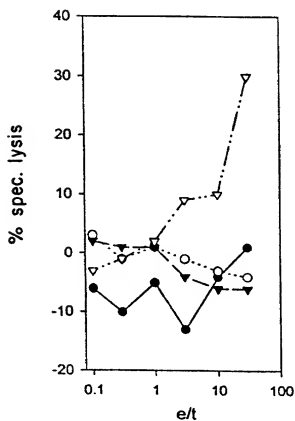
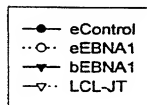


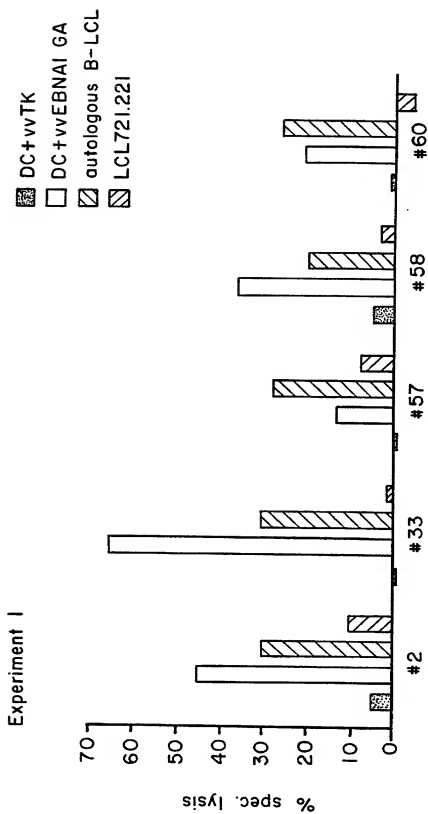
FIG. 5F

DC targets:



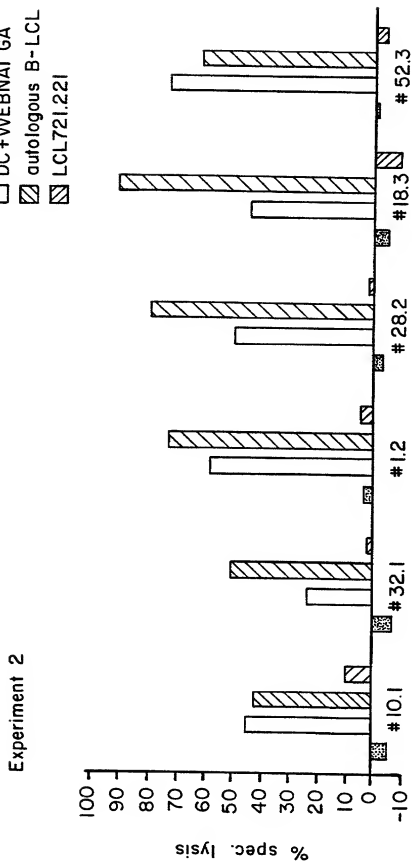
13/24

FIG. 6A



14/24

FIG. 6B



15/24

FIG. 7A

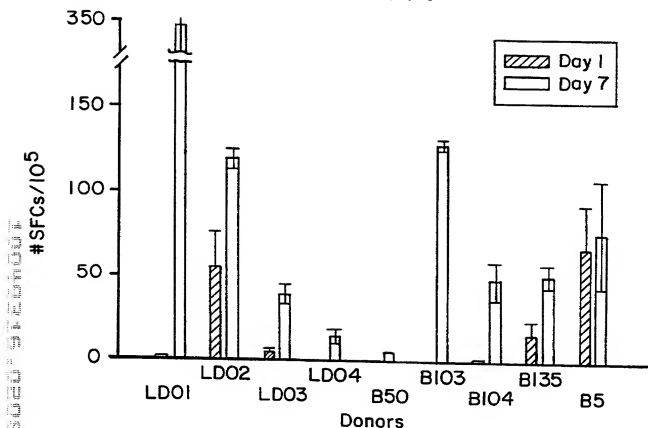


FIG. 7B

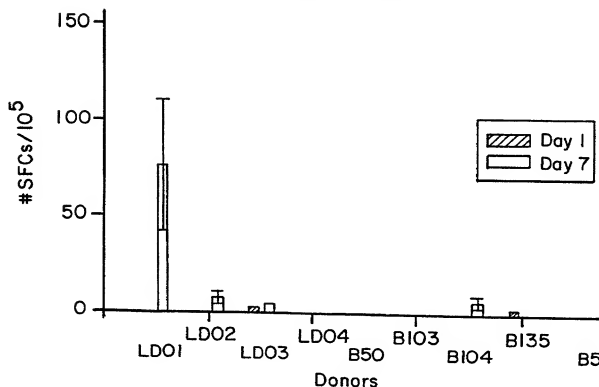
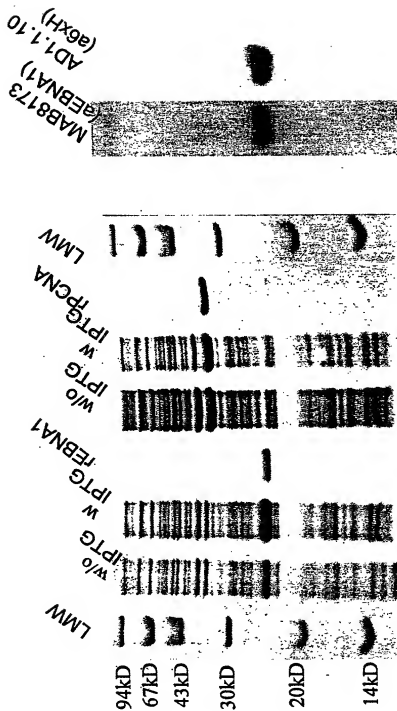


FIG. 8A



17/24

FIG. 8B

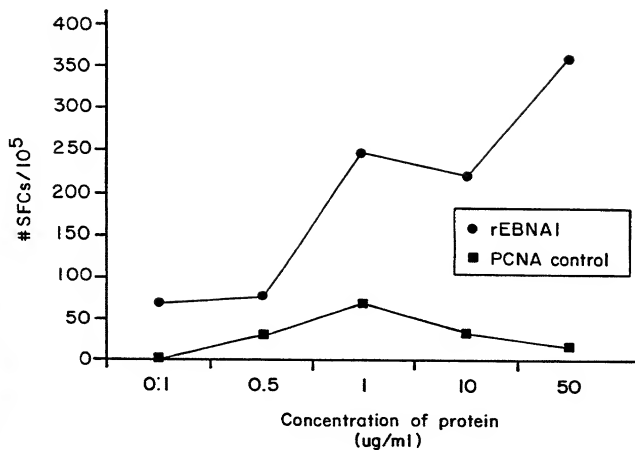
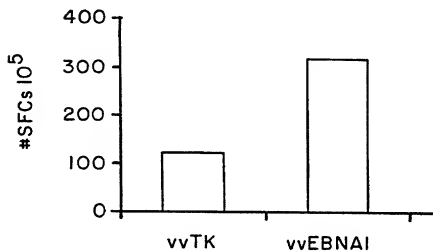


FIG. 8C



18/24

FIG. 9A

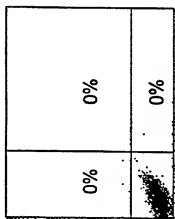


FIG. 9B

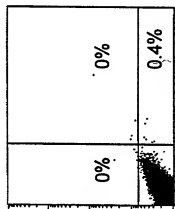
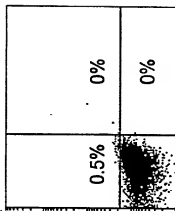


FIG. 9C



Mouse IgG2a PE

FIG. 9D

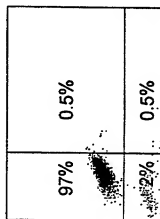
CD4
FITC

FIG. 9E

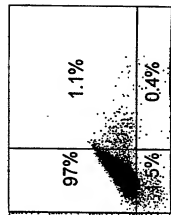
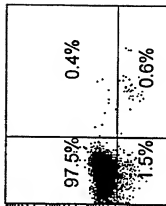


FIG. 9F



CD8 PE

19/24

FIG. 10A

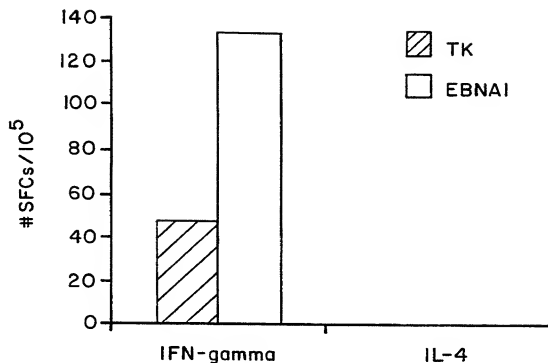
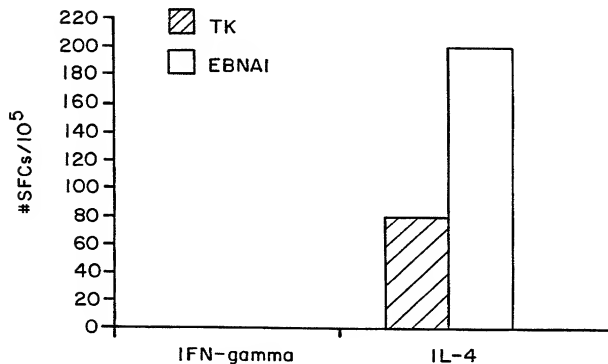


FIG. 10B



20/24

FIG. 10C

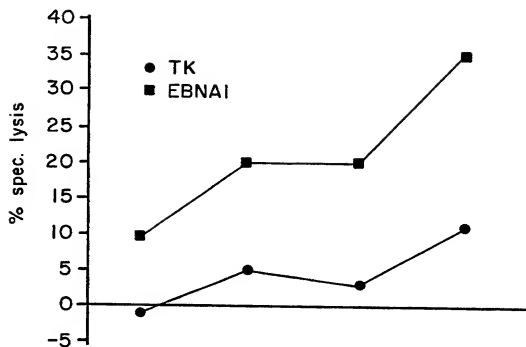
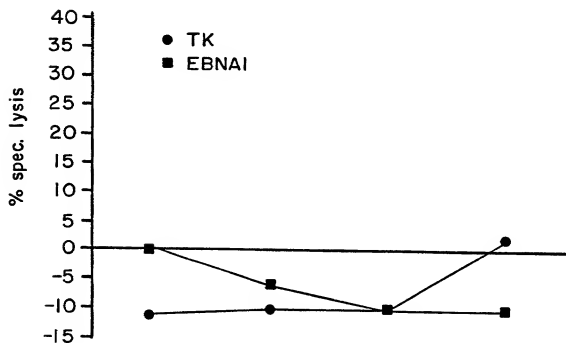


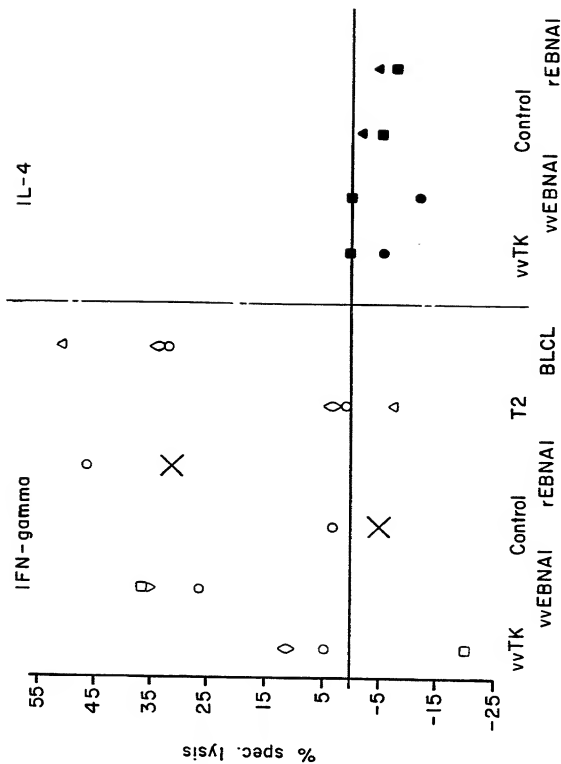
FIG. 10D



21/24

SUBSTITUTE SHEET (RULE 26)

FIG. 10E



22/24

FIG. IIA

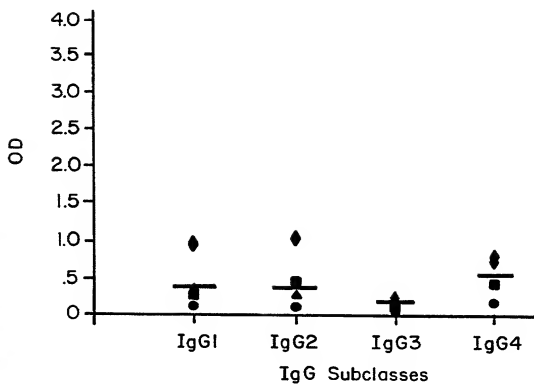
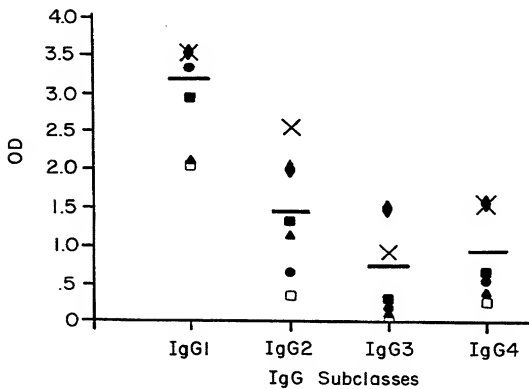


FIG. IIB



23/24

FIG. IIC

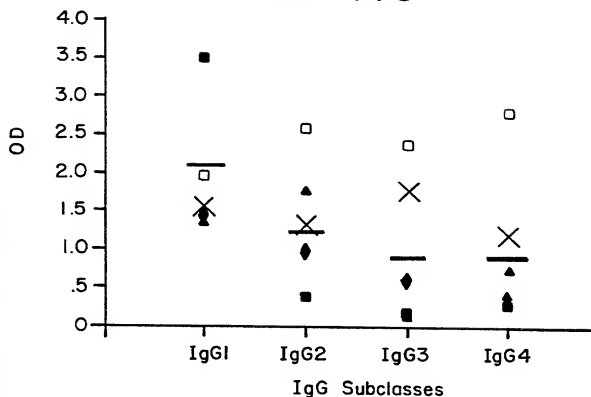
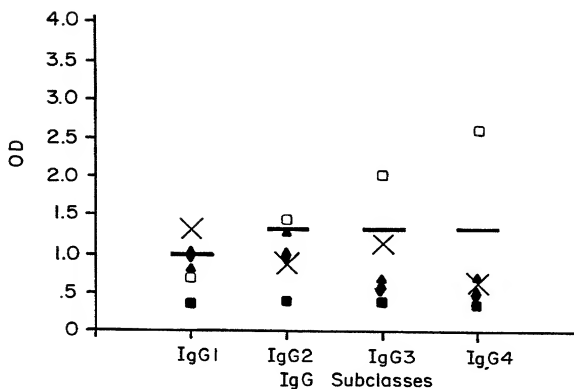
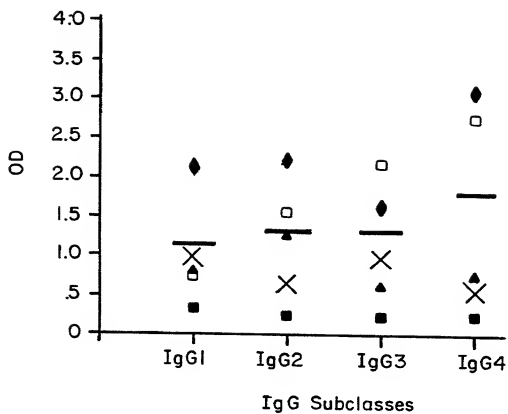


FIG. IID



24/24

FIG. 11E



COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

(Includes Reference to PCT International Applications)

ATTORNEY DOCKET NUMBER
7529/1F590-US1

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed for and which a patent is sought on the invention entitled:

PROTECTIVE ANTIGEN OF EPSTEIN BARR VIRUS

the specification of which (check only one item below):

☐ is attached hereto.☐ was filed as United States application

Serial No. _____

on _____

and was amended

on _____ (if applicable).

☒ was filed as PCT international applicationNumber PCT/US00/22106on 10 August 2000

and was amended under PCT Article 19

on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. 119
United States	60/148,971	13 August 1999	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

STATUS (Check one)

ABANDONEC

U.S. SERIAL NUMBER
ASSIGNED (if any)

Direct Telephone Calls to:
(name and telephone number)

Paul F. Fehlner, Ph.D.
(212) 527-7700

SIGNATURE OF INVENTOR 1B		SIGNATURE OF INVENTOR 2B		SIGNATURE OF INVENTOR 3B	
<i>Phil Stinson</i>		<i>Christian Miller</i>			
DATE 02/04/02		DATE 02/04/02		DATE	